The Effect of Photodynamic Therapy Using 5-Aminolevulinic Acid in Bone and Soft Tissue Sarcoma Cells

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Experimental study

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Abstract

Background/Aim: 5-Aminolevulinic acid (5-ALA) is a natural amino acid and a precursor of protoporphyrin IX (PpIX). Following light irradiation, the PpIX generates reactive oxygen species (ROS) in the presence of oxygen. Increased ROS levels can cause apoptotic cell death and necrosis of targeted cancer cells. This study examined whether photodynamic therapy using 5ALA (5-ALA PDT) could be used as a potential adjuvant therapy for bone and soft tissue sarcomas.

Materials and Methods: The human osteosarcoma (143B), mouse osteosarcoma (LM8), human fibrosarcoma cell (HT1080) cell lines were used. *In vitro*, cultured cells were exposed to 5-ALA at various concentrations followed by strobe scope light irradiation for 10 min as 5-ALA PDT. Cell viability was then measured. *In vivo*, each tumour cell line was inoculated subcutaneously into the backs of mice. In the 5-ALA PDT group, 5-ALA (250 mg/kg) was administered intraperitoneally followed by light irradiation. Change in tumour volume by 5-ALA PDT were primarily evaluated.

Results: *In vitro*, treatment of sarcoma cells with 100 and 200 μg/ml 5-ALA PDT significantly inhibited cell proliferation at 24 and 48h, compared with the group treated with 0 and 10 μg/ml 5-ALA PDT. *In vivo,* in all cell lines, a significant inhibition of the tumour volume was observed in the 5-ALA-PDT group as compared to that in control, strobe scope light, and 5-ALA groups.

Conclusion: 5-ALA PDT effectively inhibited proliferation of bone and soft tissue sarcoma cell lines. Further in vivo research using other subtypes of bone and soft tissue sarcoma is warranted to confirm the applicability in clinical setting.

Photodynamic therapy (PDT) is a minimally invasive therapy that induces tissue damage using light, after the administration of a photosensitizer (PS), which can be selectively retained in malignant lesions relative to normal tissues (1, 2). PDT is based on the activation of the PS upon light irradiation at a specific wavelength (3). After light irradiation, the PS generates reactive

oxygen species (ROS) in the presence of oxygen, and increased ROS levels can cause apoptotic cell death and necrosis of targeted cancer cells (2-4).

5-Aminolevulinic acid (5-ALA) is a natural amino acid and precursor of protoporphyrin IX (PpIX). When exogenous 5-ALA is taken up by normal cells, it enters the porphyrin metabolism pathway to synthesize haem. PpIX selectively accumulates in cancer cells as a PS because of the low activity of ferrochelatase (FECH), the enzyme responsible for metabolizing PpIX into haem (5, 6). The traditional excitation light sources for PDT using 5-ALA (5-ALA PDT) mainly include red (630–635 nm) and blue (approximately 410 nm) wavelengths of light. Red light is typically used for dermatological applications. Commonly used light source transmitters include light-emitting diodes, diode lasers, and intense pulsed light. Furthermore, daylight as composite light, can also be used as a light source for 5-ALA PDT (1, 3, 7-9).

Although there are several reports concerning PDT using PS in bone and soft tissue sarcomas (2, 10, 11), there are few reports concerning 5-ALA PDT use in bone and soft tissue sarcoma cell lines in vivo (12). 5-ALA is advantageous because it has already been approved for clinical cancer treatments, such as brain tumours (13). This study analyzed whether 5-ALA PDT could be used as a potential adjuvant therapy for bone and soft tissue sarcomas. Therefore, the efficacy of 5-ALA PDT was investigated in bone and soft tissue sarcoma cell lines *in vitro* and *in vivo*.

Materials and Methods

Cell lines. We used the human osteosarcoma (143B), mouse osteosarcoma (LM8), human fibrosarcoma cell (HT1080), and mouse myoblast (C2C12) cell lines *in vitro* and *in vivo*. The 143B, LM8, and HT1080 cells were cultured in minimum essential medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS), and the C2C12 cell line was cultured in Dulbecco's modified Eagle's minimum essential medium (Gibco, Carlsbad, CA, USA) containing 15% FBS. The cells were maintained as attached monolayers and incubated in a humidified atmosphere with 5% CO_2 at 37°C.

***In vitro* study.**

Accumulation of PpIX in C2C12 and HT1080 cells. The C2C12 cell line was used as a control group for comparison with cancer cell lines. C2C12 and HT1080 cells were seeded (5×10^3 cells/well) in 96-well plates with 50 μl of FBS-free medium and incubated at 37°C in a 5% CO_2 atmosphere. All experiments were conducted after 24 h of culturing. The cells were exposed to different concentrations of 5-ALA (0 μg/ml, 10 μg/ml, 100 μg/ml, and 200 μg/ml) (FUJIFILM WAKO Chemicals Corporation, Kanagawa, Japan). The accumulation of PpIX in C2C12 and HT1080 cells was assessed at 0, 24, and 48 h (n=21). PpIX fluorescence was quantified using a

microplate reader (PerkinElmer, Yokohama, Japan; excitation: 406 nm, emission: 620 nm) to measure PpIX accumulation.

Western blot analysis. The 143B, LM8, HT1080, and C2C12 cells were lysed using radioimmunoprecipitation buffer (Millipore-Upstate, Temecula, CA, USA) supplemented with a protease inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na_3VO_4. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the samples were adjusted to the same protein concentration before loading. The proteins were transferred onto nitrocellulose membranes and blotted. Antibodies were obtained from the following sources and used at the dilutions recommended by the manufacturer: FECH (1:500 dilution; Arigo Biotechnology, Hsinchu City, Taiwan) and β-actin (1:500 dilution; Abcam, Cambridge, UK) was used as a loading control.

MTS assay. The 143B, LM8, and HT1080 cells were seeded (5×10^3 cells/well) in 96-well plates with 50 μL FBS-free medium and incubated at 37°C in a 5% CO_2 atmosphere. All experiments were conducted after 24 h of culturing. The cells were then divided into four groups (n = 6 per group). (Group 1: 5-ALA-free medium; Group 2: 10 μg/ml 5-ALA; Group 3: 100 μg/ml 5-ALA; Group 4: 200 μg/ml 5-ALA, n=6 per group). At the beginning of treatment (0 h), the medium in each well was supplemented with 50 μl of FBS-free medium containing different concentrations

of 5-ALA (10, 100, or 200 μg/ml) or 50 μl FBS-free medium. Strobe scope light (the light illumination frequency was 60 Hz and the pulse width was 1 ms, the energy was 15 J, and the illumination level was 1,000,000 lux.) were exposed for 10 min after administration of 5-ALA. The viability of 143B, LM8, and HT1080 cells in each well was assessed at 0, 12, 24, and 48 h using the Cell Titre 96 Aqueous One Solution Cell Proliferation Assay (G3581; Promega Corporation, Madison, Wisconsin, USA).

***In vivo* study.** The 143B, LM8, and HT1080 cells (2×10^6) were isolated by trypsinization and inoculated into the backs of BALBC mice (five-week-old males). After tumours were macroscopically visible (5 mm in diameter), the following experiments were performed: The tumour volume (V) was calculated using the following equation: $V = \pi \times (\text{major axis}) \times (\text{minor axis})2 /6$ (14). The following four groups were generated (each group, n = 5): the control group was not treated, and the treatment groups were illuminated with strobe scope light alone for 10 min (PTD group), intraperitoneal administration of 5-ALA at 250 mg/kg alone (5-ALA group), and 5-ALA administration followed by illumination with strobe scope light for 10 min (5-ALA PDT) group). PDT was performed after 2 h from administration of 5-ALA. Subsequently, body weight and tumour diameter were measured twice per week. The mice were sacrificed on the 14th

day after treatment and the tumour tissues were resected for evaluation in all groups. All mice in each group survived for 14 days, up to the study endpoint.

To evaluate the effect on lung metastasis, the excised lungs were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin for histological observation. The number of lung metastases was counted under a high-power light microscope based on histological findings, regardless of tumour size, which was independently reviewed by a pathology technician (TI) and orthopaedic surgeons (TN, TH, and TU).

Statistical analysis. All *in vitro* experiments were repeated at least three times to ensure reproducibility. Data are expressed as the mean ± standard deviation (SD). Significant differences between the two groups were compared using the Mann–Whitney U test, and more than three groups were compared using the Kruskal–Wallis test. Statistical significance was set at $p<0.05$. All statistical analyses were performed using the EZR graphical user interface (Saitama Medical Centre, Jichi Medical University, Saitama, Japan) for the R package (R Foundation for Statistical Computing, Vienna, Austria), which is a modified version of R Commander designed to add statistical functions commonly used in biostatistics.

Ethics statement. The experiments were performed in accordance with the Declaration of Helsinki and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research,

Testing, and Education. The study was approved by the Ethics Committee of the Animal Research Committee of our institution (approval number 2022-12).

Results

Accumulation of PpIX in C2C12 and HT1080 cells. Treatment of C2C12 and HT1080 groups with 100 μg/ml and 200 μg/ml 5-ALA for 24 h and 48 h resulted in an increase in the accumulation of PpIX increased in a time-dependent manner (Figure 1). When we compared the C2C12 and HT1080 groups, the HT1080 group showed a significantly higher accumulation of PpIX at 24 h and 48 h after treatment ($p<0.01$, $p<0.01$, respectively; Mann–Whitney U test) (Figure 1A and B).

Western blot analysis. Western blotting was performed to assay the protein levels of FECH, which is a haem biosynthesis enzyme, in osteosarcoma and fibrosarcoma cell lines. FECH protein levels were higher in C2C12 cells than those in LM8, 143B and HT1080 cells (Figure 2).

MTS assay. Cell proliferation was evaluated at each concentration of 5-ALA after PDT using a cell proliferation assay. Treatment of 143B cells with 100 and 200 μg/ml 5-ALA PDT for 24 and 48h significantly reduced proliferation compared with the control group and that treated with 10 μg/ml 5-ALA PDT (Figure 3A).

In the LM8 cells, proliferation in the 100 and 200 μg/ml 5-ALA PDT groups was significantly inhibited at 24 and 48 h, compared with the 0 and 10 μg/ml 5-ALA PDT groups (Figure 3B).

In the HT1080 cells, 48 h after treatment, a significant decrease in cell proliferation was observed in the 100 μg/ml 5-ALA PDT and 200 μg/ml groups, compared with that in the 0 and 10 μg/ml 5-ALA PDT groups (Figure 3C).

Mouse xenograft model. During the 14 days after treatment, there were no obvious differences in body weight between the groups. In the mice inoculated with 143B cells the 5-ALA PDT group exhibited a significant reduction in tumour volume compared to that of the control, 5-ALA alone, and strobe scope light alone groups (Figure 4A). The mean tumour size of the 5-ALA PDT group at 14 days was 156.6 mm^3 (SD: 63.1, range=101–265.3 mm^3), whereas the mean tumour sizes of the control, 5-ALA alone, and strobe scope light groups were 452.5 mm^3 (SD: 108.6, range=329.3–605.4 mm^3), p=0.008; 407.9 (SD: 81.4, range=298.4–533.2) mm^3, p=0.008; and 484 mm^3 (SD: 118.3, range=348.6–643.4 mm^3), p=0.008; all Mann–Whitney U test, respectively (Figure 4A).

In mice inoculated with LM8 cells, the tumour volume in the 5-ALA PDT group was significantly lower than those in the other three groups. The mean tumour size of the 5-ALA PDT group at 14 days was 423.9 mm^3 (SD: 86.8, range=298.5–539.7 mm^3), whereas the mean

tumour sizes of the control, 5-ALA alone, and strobe scope light alone groups were 761.2 mm^3 (SD: 145.9, range=598.6–987.4 mm^3), p=0.008; 666.3 (SD: 141.8, range=463.6–824.6 mm^3), p=0.016 and 716.6 mm^3 (SD: 126.7, range=550.4–842 mm^3), p=0.008; all Mann–Whitney U test, respectively (Figure 4B).

In mice inoculated with HT1080 cells, there was a significant difference in the tumour volume between the 5-ALA PDT group and the other three groups (Figure 4C). The mean tumour size of 5-ALA PDT group at 14 days was 473.4 mm^3 (SD: 103, range=400.8–631.9 mm^3), whereas the mean tumour sizes of the control, 5-ALA alone and strobe scope light groups were 627.4 mm^3 (SD: 92.7; range=522.4–689.4 mm^3), p=0.032), 615.6 (SD: 90.6, range=509.8–702 mm^3), p=0.048 and 684.6 mm^3 (SD: 96, range=599.4–844.2, p=0.021; all Mann–Whitney U test, respectively.

Analysis of lung metastasis. In the LM8 and HT1080 xenograft models, there were significant differences between the control and treatment groups with 5-ALA PDT (p=0.034 for LM8 and p=0.038 for HT1080; Mann–Whitney U test). In the 143 B xenograft model, there were no lung metastases in the control or treatment groups with 5-ALA PDT.

Discussion

We first demonstrated the efficacy of 5-ALA PDT in osteosarcoma and fibrosarcoma cells *in vitro* and *in vivo*. The United States Food and Drug Administration (FDA) has approved PDT for clinical applications in oncology and other diseases (7, 13). Our results may contribute to the application of 5-ALA PDT in the field of bone and soft tissue sarcomas.

To evaluate PpIX accumulation in sarcoma cells, we compared PpIX accumulation in C2C12 and HT1080 cell lines. A previous study indicated that PpIX is formed more abundantly in tumour tissues than in normal tissues after systemic distribution of 5-ALA (15). We also confirmed the higher accumulation of PpIX in HT1080 cells than in C2C12 cells. We also evaluated the expression of FECH using western blot assay because PpIX selectively accumulates in cancer cells owing to the low activity of FECH (16). FECH protein levels in all three tumour cell lines were lower than those in C2C12 cells. Therefore, FECH also plays an essential role in the regulation of PpIX levels in osteosarcoma and fibrosarcoma cell lines.

In both *in vitro* and *in vivo* studies, we demonstrated the anti-tumour effects of 5-ALA PDT. Surgical tumour resection with adequate margins is the standard treatment for bone and soft

tissue sarcoma (17, 18). However, when tumours are close to major vessels, nerves, and bones, patients may develop serious dysfunction of the affected limb after wide resection. Amputation may be required in some patients (19). Although adjuvant radiotherapy may be effective in reducing local recurrence, fibrosis, oedema, radiation-induced fractures, and contractures may also occur (2, 20, 21). Therefore, it is imperative to urgently develop new adjuvant therapies to reduce the local recurrence rate after marginal or intralesional tumour resection, without damaging adjacent normal tissues in patients with high-grade soft tissue sarcomas, in order to maintain excellent postoperative limb function. Our results suggest that 5-ALA PDT may be a useful option for the adjuvant treatment of bone and soft tissue sarcomas. In addition to anti-cancer treatment, 5-ALA photodynamic diagnosis (PDD) has been widely used in recent years to enable fluorescence diagnosis of brain tumours and bladder cancer because tumour tissues specifically emit red fluorescence at approximately 635 nm after 5-ALA administration with blue light excitation at 405 nm (22). 5-ALA PDT has been employed for palliative and curative treatment of endoscopically accessible tumours (1). Lung, oesophageal, gastric, and bladder carcinomas, as well as oral premalignant lesions, gynaecological intraepithelial neoplasias, and Barrett's oesophagus, are the conditions mostly treated with PDT using 5-ALA (1, 23). However, owing to the limited penetration of ALA and light, the non-dermatological uses of ALA-PDT have not progressed beyond phase I clinical trials (1). Regarding bone and soft tissue sarcomas,

5-ALA PDD and PDT can be applied in the surgical field to observe the fluorescence of PpIX following excitation with a blue light beam using a surgical fluorescence microscope, which is similar to the procedure used to treat brain tumours using 5-ALA PDT.

Finally, we evaluated lung metastases *in vivo*. There have been no reports regarding the inhibition of pulmonary metastases *in vitro*. In the present study, PDT with 5-ALA treatment significantly inhibited lung metastases in mice inoculated with LM8 and HT1080 cell lines. There are two possible explanations for this finding. First, because the mice treated with 5-ALA PDT received illumination over the entire body, the high-power flash wave light penetrated the chest wall, exerting a cytocidal effect against metastatic lesions that had incorporated the intravenously administered 5-ALA. Second, this effect may largely be due to growth inhibition of the primary tumour. Further studies are required to confirm the effects of 5-ALA PDT on lung metastasis.

Conclusion

5-ALA PDT was effective in the 143B, LM8, and HT1080 cell lines. Further in vivo research using other subtypes of bone and soft tissue sarcoma is warranted to confirm the applicability in clinical setting.

Conflicts of Interest

The Authors declare no conflict of interest in the present study.

Authors' Contributions

Tomoki Nakamura: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resource, Data curation, Writing-original draft, Visualization, Project administration, Funding acquisition. Ryohei Adachi: Methodology, Software, Resource, Data curation, Formal analysis, Investigation, Writing-original draft. Kenta Nakata: Validation, Resource, Data curation Teruya Uchiyama: Validation, Resource, Data curation Tomohito Hagi: Formal analysis, Resource, Investigation, Kunihiro Asanuma: Project administration , Resource, Data curation, Supervision. Akihiro Sudo: Project administration, Funding acquisition, Supervision, Writing-editing & review.

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References

1. Casas A: Clinical uses of 5-aminolaevulinic acid in photodynamic treatment and photodetection of cancer: A review. Cancer Lett 490: 165-173, 2000. DOI: 10.1016/j.canlet.2020.06.008
2. Nakamura T, Kusuzaki K, Matsubara T, Murata H, Hagi T, Asanuma K, Sudo A: Long-term clinical outcome in patients with high-grade soft tissue sarcoma who were treated with surgical adjuvant therapy with acridine orange after intra-lesional and marginal resection. Photodiagnosis Photodyn Ther 23: 165-170, 2018. DOI: 10.1016/j.pdpdt.2018.06.001
3. Guney Eskiler G, Deveci Ozkan A, Sozen Kucukkara E, Kamanlı AF, Gunoğlu B, Yıldız MZ: Optimization of 5-aminolevulinic acid based photodynamic therapy protocol for breast cancer cells. Photodiagnosis Photodyn Ther 31: 101854, 2020. DOI: 10.1016/j.pdpdt.2020.101854
4. Dolmans DE, Fukumura D, Jain RK: Photodynamic therapy for cancer. Nat Rev Cancer 3(5): 380-387, 2003. DOI: 10.1038/nrc1071

5. Koizumi N, Harada Y, Minamikawa T, Tanaka H, Otsuji E, Takamatsu T: Recent advances in photodynamic diagnosis of gastric cancer using 5-aminolevulinic acid. World J Gastroenterol 22(3): 1289-96, 2016. DOI: 10.3748/wjg.v22.i3.1289

6. Harada Y, Murayama Y, Takamatsu T, Otsuji E, Tanaka H: 5-aminolevulinic acid-induced protoporphyrin IX fluorescence imaging for tumor detection: Recent advances and challenges. Int J Mol Sci 23(12): 6478, 2022. DOI: 10.3390/ijms23126478

7. Shi L, Wang H, Chen K, Yan J, Yu B, Wang S, Yin R, Nong X, Zou X, Chen Z, Li C, Chen L, Zhang C, Zhang F, Zheng H, Zheng M, Tu P, Xu J, Tao J, Kang X, Zeng K, Lu Y, Yu N, Lei X, Pan M, Ju Q, Gu H, Wang X: Chinese guideline on the clinical application of 5-aminolevulinic acid-based photodynamic therapy in dermatology (2021 edition). Photodiagnosis Photodyn Ther 35: 102340, 2021. DOI: 10.1016/j.pdpdt.2021.102340

8. Inoue K: 5-aminolevulinic acid-mediated photodynamic therapy for bladder cancer. Int J Urol 24 (2): 97-101, 2017. DOI: 10.1111/iju.13291

9. Yamamoto S, Fukuhara H, Seki H, Kawada C, Nakayama T, Karashima T, Ogura S, Inoue K: Predictors of therapeutic efficacy of 5-aminolevulinic acid-based photodynamic therapy in human prostate cancer. Photodiagnosis Photodyn Ther 35: 102452, 2021. DOI: 10.1016/j.pdpdt.2021.102452

10. Zhang X, Liu T, Li Z, Zhang X: Progress of photodynamic therapy applications in the

treatment of musculoskeletal sarcoma (Review). Oncol Lett 8 (4): 1403-1408, 2014. DOI: 10.3892/ol.2014.2332

11. Sun J, Xing F, Braun J, Traub F, Rommens PM, Xiang Z, Ritz U: Progress of phototherapy applications in the treatment of bone cancer. Int J Mol Sci 22: 11354, 2021. DOI: 10.3390/ijms222111354

12. Guder WK, Hartmann W, Buhles C, Burdack M, Busch M, Dunker N, Hardes J, Dirksen U, Bauer S, Streitburger A: 5-ALA-mediated fluorescence of musculoskeletal tumors in a chick chorio-allantoic membrane model: preclinical in vivo qualification analysis as a fluorescence-guided surgery agent in Orthopedic Oncology. J Orthop Surg Res 17 (1): 34, 2022. DOI: 10.1186/s13018-022-02931-x

13. Mahmoudi K, Garvey KL, Bouras A, Cramer G, Stepp H, Jesu Raj JG, Bozec D, Busch TM, Hadjipanayis CG: 5-aminolevulinic acid photodynamic therapy for the treatment for high-grade gliomas. J Neurooncol 141(3): 595-607, 2019. DOI: 10.1007/s11060-019-03103-4

14. Ovejera AA, Houchens DP, Barker AD: Chemotherapy of human tumor xenografts in genetically athymic mice. Ann Clin Lab Sci 8 (1): 50–56, 1978.

15. Nakanishi T, Ogawa T, Yanagihara C, Tamai I: Kinetic Evaluation of Determinant Factors for Cellular Accumulation of Protoporphyrin IX Induced by External 5-Aminolevulinic Acid for Photodynamic Cancer Therapy. J Pharm Sci 104 (9): 3092-3100, 2015. DOI:

10.1002/jps.24462

16. Fukuhara H, Inoue K, Kurabayashi A, Furihata M, Fujita H, Utsumi K, Sasaki J, Shuin T: The inhibition of ferrochelatase enhances 5-aminolevukinic acid-based photodynamic action for prostate cancer. Photodiagnosis Photodyn Ther 10 (4): 399-409, 2013. DOI: 10.1016/j.pdpdt.2013.03.003

17. Gronchi A, Miah AB, Dei Tos AP, Abecassis N, Bajpai J, Bauer S, Biagini R, Bielack S, Blay JY, Bolle S, Bonvalot S, Boukovinas I, Bovee JVMG, Boye K, Brennan B, Brodowicz T, Buonadonna A, De Álava E, Del Muro XG, Dufresne A, Eriksson M, Fagioli F, Fedenko A, Ferraresi V, Ferrari A, Frezza AM, Gasperoni S, Gelderblom H, Gouin F, Grignani G, Haas R, Hassan AB, Hecker-Nolting S, Hindi N, Hohenberger P, Joensuu H, Jones RL, Jungels C, Jutte P, Kager L, Kasper B, Kawai A, Kopeckova K, Krákorová DA, Le Cesne A, Le Grange F, Legius E, Leithner A, Lopez-Pousa A, Martin-Broto J, Merimsky O, Messiou C, Mir O, Montemurro M, Morland B, Morosi C, Palmerini E, Pantaleo MA, Piana R, Piperno-Neumann S, Reichardt P, Rutkowski P, Safwat AA, Sangalli C, Sbaraglia M, Scheipl S, Schöffski P, Sleijfer S, Strauss D, Strauss S, Sundby Hall K, Trama A, Unk M, van de Sande MAJ, van der Graaf WTA, van Houdt WJ, Frebourg T, Casali PG, Stacchiotti S; ESMO Guidelines Committee, EURACAN and GENTURIS: Soft tissue and visceral sarcoma: ESMO-EURACAN-GENTURIS clinical practice guidelines for diagnosis,

treatment and follow-up. Ann Oncol 32 (11) 1348-65, 2021. doi: 10.1016/j.annonc.2021.07.006

18. Strauss SJ, Frezza AM, Abecassis N, Bajpai J, Bauer S, Biagini R, Bielack S, Blay JY, Bolle S, Bonvalot S, Boukovinas I, Bovee JVMG, Boye K, Brennan B, Brodowicz T, Buonadonna A, de Álava E, Dei Tos AP, Garcia Del Muro X, Dufresne A, Eriksson M, Fagioli F, Fedenko A, Ferraresi V, Ferrari A, Gaspar N, Gasperoni S, Gelderblom H, Gouin F, Grignani G, Gronchi A, Haas R, Hassan AB, Hecker-Nolting S, Hindi N, Hohenberger P, Joensuu H, Jones RL, Jungels C, Jutte P, Kager L, Kasper B, Kawai A, Kopeckova K, Krákorová DA, Le Cesne A, Le Grange F, Legius E, Leithner A, López Pousa A, Martin-Broto J, Merimsky O, Messiou C, Miah AB, Mir O, Montemurro M, Morland B, Morosi C, Palmerini E, Pantaleo MA, Piana R, Piperno-Neumann S, Reichardt P, Rutkowski P, Safwat AA, Sangalli C, Sbaraglia M, Scheipl S, Schöffski P, Sleijfer S, Strauss D, Sundby Hall K, Trama A, Unk M, van de Sande MAJ, van der Graaf WTA, van Houdt WJ, Frebourg T, Ladenstein R, Casali PG, Stacchiotti S; ESMO Guidelines Committee, EURACAN, GENTURIS and ERN PaedCan:Bone sarcomas: ESMO-EURACAN-GENTURIS-ERN clinical practice guideline for diagnosis, treatment and follow-up. Ann Oncol 32 (11): 1520-1536, 2021. DOI: 10.1016/j.annonc.2021.08.1995

19. Hagi T, Nakamura T, Nagano A, Koike H, Yamada K, Aiba H, Fujihara N, Wasa J, Asanuma

K, Kozawa E, Ishimura D, Kawanami K, Izubuchi Y, Shido Y, Sudo A, Nishida Y: Clinical outcome in patients who underwent amputation due to extremity soft tissue sarcoma: Tokai Musculoskeletal Oncology Consortium study. Jpn J Clin Oncol 52(2): 157-162, 2022. DOI: 10.1093/jjco/hyab184

20. Fuchs B, Davis AM, Wunder JS, Bell RS, Masri BA, Isler M, Turcotte R, Rock MG: Sciatic nerve resection in the thigh: a functional evaluation. Clin Orthop Relat Res 382: 34-41, 2001. DOI: 10.1097/00003086-200101000-00007

21. Ghert MA, Davis AM, Griffin AM, Alyami AH, White L, Kandel RA, Ferguson P, O'Sullivan B, Catton CN, Lindsay T, Rubin B, Bell RS, Wunder JS: The surgical and functional outcome of limb-salvage surgery with vascular reconstruction for soft tissue sarcoma of the extremity. Ann Surg Oncol 12(12): 1102-1110, 2005. DOI: 10.1245/ASO.2005.06.036

22. Nishimura M, Murayama Y, Harada K, Kamada Y, Morimura R, Ikoma H, Ichikawa D, Fujiwara H, Okamoto K, Otsuji E: Photodynamic diagnosis of hepatocellular carcinoma using-5-aminolevulinic acid. Anticancer Res 36 (9): 4569-4574, 2016. DOI: 10.21873/anticanres.11005

23. Bartusik-Aebisher D, Osuchowski M, Adamczyk M, Stopa J, Cieślar G, Kawczyk-Krupka A, Aebisher D. Advancements in photodynamic therapy of esophageal cancer. Front Oncol

12: 1024576, 2022. DOI: 10.3389/fonc.2022.1024576

Figure Legends

Figure 1. The graph shows accumulation of protoporphyrin IX (PpIX) in C2C12 and HT1080 cells after 24 h (A) and 48 h (B) of treatment. The accumulation of PpIX in HT 1080 is significantly higher than that in C2C12 after 24 h (A) and 48 h (B) of treatment (**p<0.01). (Black bar; C2C12, Gray bar; HT1080).

Figure 2. Western blotting shows weak expression of ferrochelatase (FECH) in sarcoma cell lines compared to that in C2C12 cells.

Figure 3. The cell proliferation assay showed significant inhibition of cell proliferation in the 100 and 200 μg/ml 5-Aminolevulinic acid (5-ALA) photodynamic therapy (PDT) group groups compared to the group treated with 0 μg/mL 5-ALA PDT at 48 h in 143B (A), LM8 (B), and HT1080 (C) (*p<0.05). (A: 0 μg/ml 5-ALA, B: 10 μg/ml 5-ALA, C: 100 μg/ml 5-ALA, D: 200 μg/ml 5-ALA).

Figure 4. The 5-Aminolevulinic acid (5-ALA) photodynamic therapy (PDT) group shows a significant inhibition in tumour volume compared with that in the control, 5-ALA alone, and strobe scope light alone groups at 14 days after treatment (A: 143B cells, B: LM8 cells, C: HT1080cells) (*p<0.05, **p<0.01). (A: control, B: 250 mg/kg 5-ALA, C: strobe scope light, D: 5-ALA PDT).

Figure. 1A

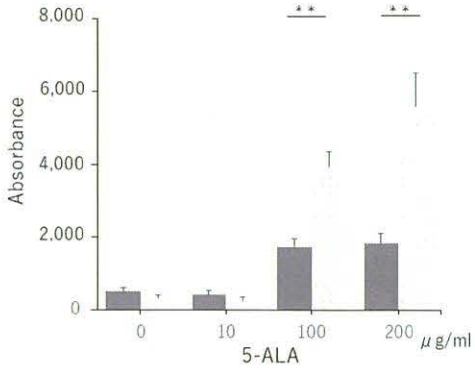


Figure. 1B

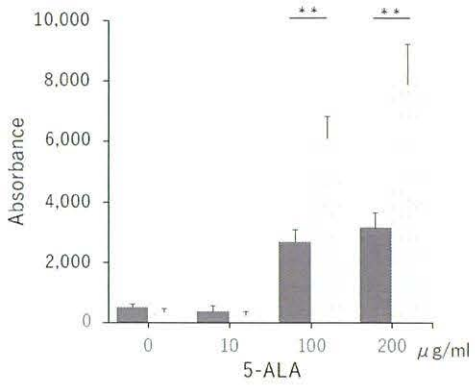


Figure. 2

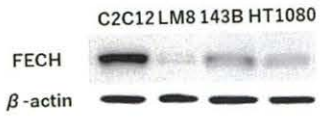


Figure. 3A

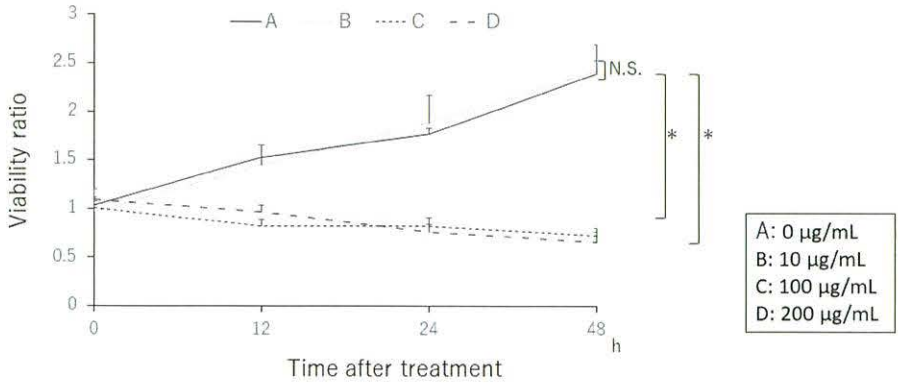

A
B
C
D
Viability ratio
3
2.5
2
1.5
1
0.5
0
0
12
24
48
h
Time after treatment
N.S.
*
*
A: 0 μg/mL
B: 10 μg/mL
C: 100 μg/mL
D: 200 μg/mL

Figure. 3B

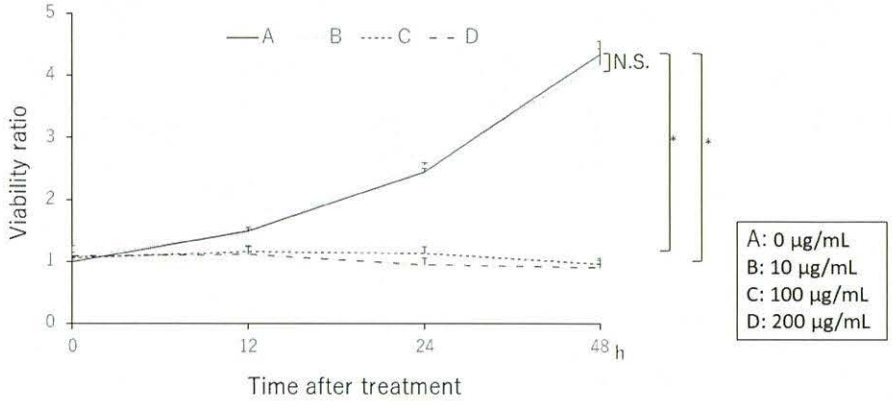

A
B
C
D
N.S.
Viability ratio
0
1
2
3
4
5
0
12
24
48 h
Time after treatment
A: 0 μg/mL
B: 10 μg/mL
C: 100 μg/mL
D: 200 μg/mL

Figure. 3C

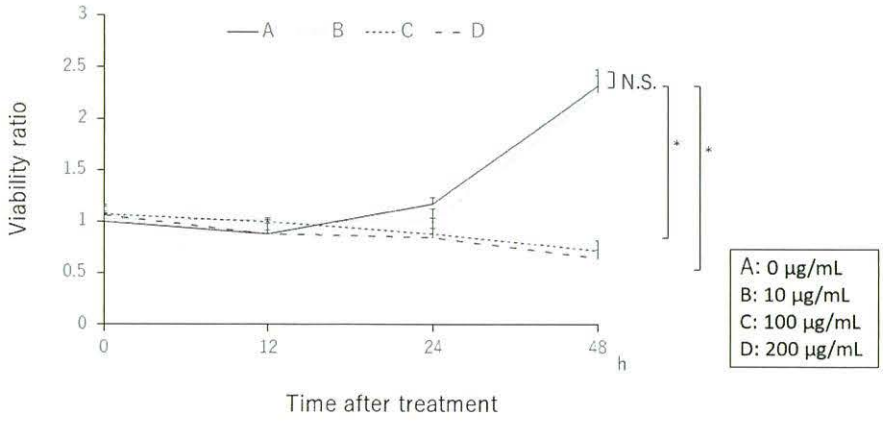

A
B
C
D
Viability ratio
3
2.5
2
1.5
1
0.5
0
0
12
24
48
h
Time after treatment
N.S.
*
*
A: 0 μg/mL
B: 10 μg/mL
C: 100 μg/mL
D: 200 μg/mL

Figure. 4A

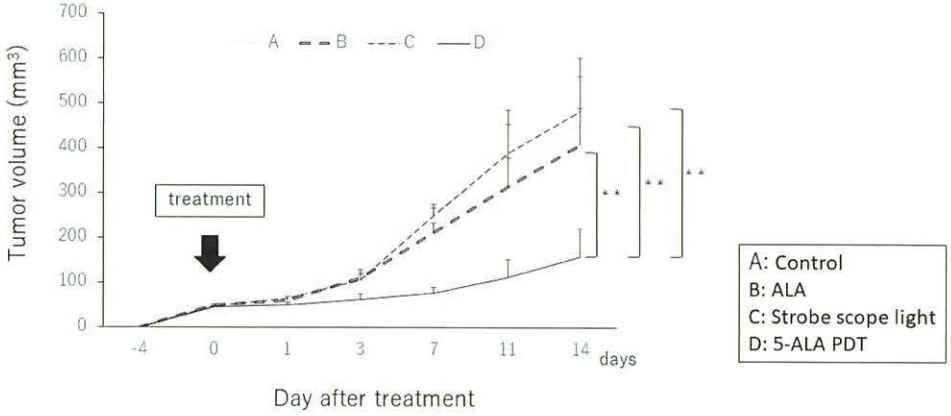

Tumor volume (mm3)
700
600
500
400
300
200
100
0
A
B
C
D
treatment
-4
0
1
3
7
11
14
days
Day after treatment
**
**
**
A: Control
B: ALA
C: Strobe scope light
D: 5-ALA PDT

Figure. 4B

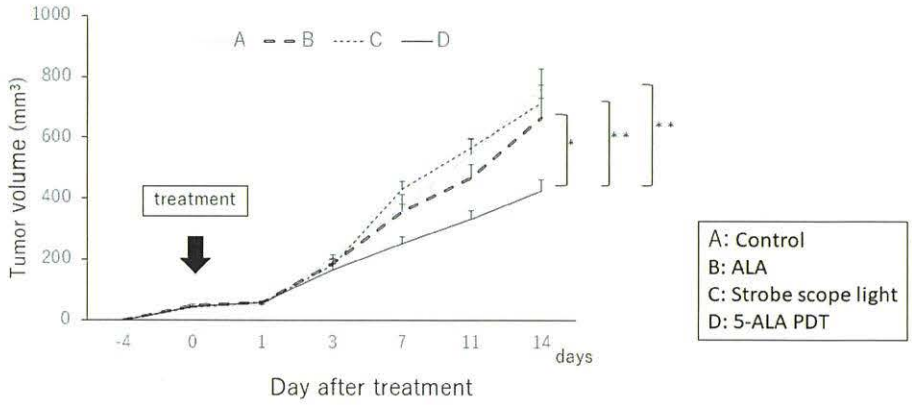
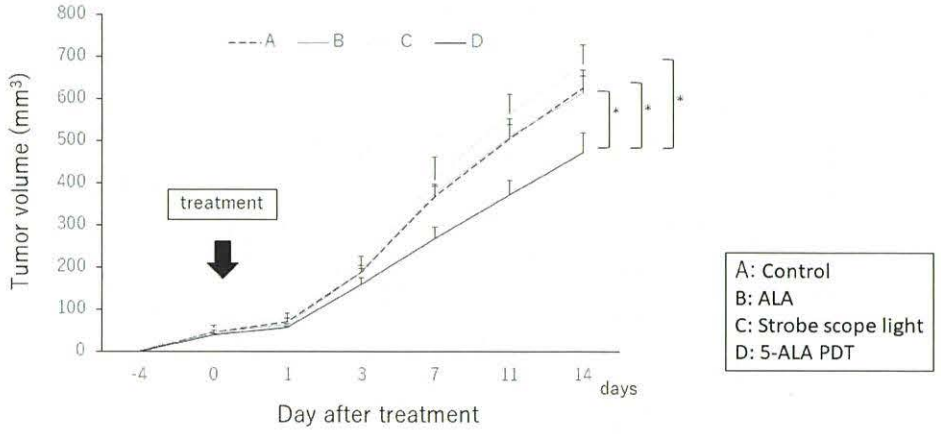

1000
800
600
400
200
0
Tumor volume (mm³)
A
B
C
D
treatment
-4
0
1
3
7
11
14
days
Day after treatment
*
**
**
A: Control
B: ALA
C: Strobe scope light
D: 5-ALA PDT

Figure. 4C


800
700
600
500
400
300
200
100
0
Tumor volume (mm³)
A
B
C
D
treatment
-4
0
1
3
7
11
14
days
Day after treatment
*
*
*
A: Control
B: ALA
C: Strobe scope light
D: 5-ALA PDT