

ALA-Porphyrin Science

Effect of 5-aminolevulinic acid and hyperthermia co-treatment on tumor growth and efficacy of low-dose anti-cancer drug *in vivo*.

Shun-ichi Ozawa¹, Tomoki Iida², Seiya Watanabe¹, Yusei Seki¹, Kiwamu Takahashi², Masahiro Ishizuka², Motowo Nakajima², and Taku Chibazakura^{1*}

1. Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan.

2. SBI Pharmaceuticals, Co., Ltd., 1-6-1 Roppongi Izumi Garden Tower 20F, Minato-ku, Tokyo 106-6020, Japan.

*Corresponding Author, Tel: 81-3-5477-2759; E-mail: taku@nodai.ac.jp

Summary

5-aminolevulinic acid (5-ALA), a starting substrate for synthesizing porphyrins including heme, has been used for photodynamic diagnosis and therapy of cancers since cancer cells specifically accumulate a photosensitizing metabolite protoporphyrin IX upon 5-ALA administration. These applications, however, bear limitations in treating cancers deep inside the patients' tissues where light irradiation is difficult. On the other hand, we have previously shown that 5-ALA enhances cell death under thermal stress in some cancer cells, suggesting a potential of 5-ALA as a sensitizer for hyperthermia of cancer. Here we examined the effect of 5-ALA and hyperthermia (ALA-HT) co-treatment on tumor growth in mouse xenograft model and found that 5-ALA significantly suppresses the tumor growth in combination with hyperthermia. However, we also observed that the tumors under the co-treatment eventually start to re-grow and histopathological analyses revealed no evidence of cell death enhancement in those tumors, implying that ALA-HT treatment is not sufficient for eliminating tumors. We then explored a potential synergy between ALA-HT treatment and a low dose of anti-cancer drug. Combination of ALA-HT treatment with a low and less-effective dose of sorafenib, a widely-used anti-cancer drug, notably enhanced the cancer cell death *in vitro* and exhibited a significantly stronger anti-proliferative effect on xenografted tumors in mice, compared to those treated with sorafenib alone. These findings suggest that ALA-HT treatment can reduce the effective dosage in cancer chemotherapy and thereby contribute to amelioration of patients' quality of life.

Keywords: 5-aminolevulinic acid (5-ALA), hyperthermia, cancer, mouse xenograft model, sorafenib (SFN)

Introduction

5-Aminolevulinic acid (5-ALA) is a natural compound present in both plant and animal cells, which is a common precursor to heme and chlorophylls [1]. Administration of 5-ALA has been shown to increase heme production, resulting in elevation of the activity of hemoproteins such as cytochrome *c* oxidase (COX). Ogura *et al.* [2] demonstrated that 5-ALA administration improves COX activity, which is decreased with aging and in various diseases, including cancer. Moreover, 5-ALA supplementation has been applied to livestock and human [3-5] and well investigated regarding the safety [6].

Besides its nutritional and metabolic effects, administration of 5-ALA has been found to enhance the cancer-specific accumulation of protoporphyrin IX (PpIX), an immediate precursor of heme [7,8]. This property of 5-ALA has been utilized clinically for the detection of malignant tissues by 5-ALA-induced PpIX fluorescence and for the selective phototoxic destruction of malignant cells, due to an increase in reactive oxygen species (ROS) derived from photo-excited PpIX, by 5-ALA-induced photodynamic therapy (ALA-PDT). The utilization of ALA-PDT has drawn significant attention in research and clinical applications, particularly in addressing a spectrum of cancers including those affecting the skin and brain [9-16], because of the appeal of 5-ALA in its notable advantages, characterized by minimal side effects, chemical stability, and brief photosensitivity compared to alternative photosensitizers [9]. Despite these merits, the practical implementation of PDT remains constrained to cancerous regions amenable to irradiation device accessibility [17].

Alternatively, hyperthermia has emerged as a widely acknowledged and practiced cancer therapy, distinguished by its minimal invasiveness and reduced side effects. Its efficacy as a sensitizer for radiotherapy and chemotherapy has positioned hyperthermia as a valuable adjunctive therapy for various cancers [18, 19]. Nevertheless, the thermal dose-response relationship exhibits variability across different cell lines and is influenced by such environmental factors as pH [20].

To improve the limitations in both PDT and hyperthermia, we have tested if 5-ALA can enhance hyperthermic effect in cancer cells and shown that the combination of 5-ALA and hyperthermia (ALA-HT) treatments enhances the cell death in certain types of cancer cell lines *in vitro* [21]. The cell death enhancement depends on relatively a high accumulation of PpIX upon 5-ALA administration and concomitant increase of ROS under the thermal stress in those cancer cells [21]. In this study, we further investigated the potential of ALA-HT as a treatment for cancer *in vivo* using tumor xenografted mouse models. We show that ALA-HT treatment significantly suppresses the tumor growth in xenografted mice, compared to hyperthermia treatment only. We also examined if ALA-HT can sensitize the tumor cells to a low dose of anti-cancer drug and demonstrate that ALA-HT can enhance the tumor suppressive effect of sorafenib, a multi-kinase inhibitor that has been used against a wide variety of tumors [22], at low doses both *in vitro* and *in vivo*. This observation particularly suggests that ALA-HT can reduce the effective dose of anti-cancer drug and possibly decrease the side effects caused by the drug such as sorafenib [23].

Experimentals

Cells and reagents

Caco-2 (human colorectal adenocarcinoma) and HepG2 (derived from human liver cancer) cells were derived from M. Nakajima's cell stock as previously described [21]. Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) with 5% CO₂. 5-aminolevulinic acid (5-ALA; hydrochloride salt) was provided by SBI Pharmaceuticals Co., Ltd, (Tokyo, Japan). Sorafenib tosylate (BAY 43-9006; BioVision, Inc., Waltham, MA, U.S.A.) was dissolved in DMSO at 1 mM as a stock solution for addition to the culture medium, or in 20% sulfolbutylether β -cyclodextrin (MedChemExpress LLC, Monmouth Junction, NJ, U.S.A.) with sonication at 10 mg/mL as a stock solution for oral administration to mice.

Mice and tumor xenografts

All xenograft experiments were performed in accordance with the guidelines of the Laboratory Animal Ethics Committee of Tokyo University of Agriculture (Tokyo, Japan). All experimental protocols were approved by the Experimental Animal Ethics Committee of Tokyo University of Agriculture (approval no.2019088).

5-week-old male BALBnu/CrlCrlj nude mice (Charles River Laboratories, Wilmington, MA, U.S.A.) were housed in a pathogen-free room at 23°C room temperature and 55±5% humidity under a 12-hour fluorescent lighting cycle and given free access to autoclaved food CE-2 (CLEA Japan, Inc., Tokyo, Japan) and sterile water for 1 week, and then used for tumor xenograft. Caco-2 or HepG2 cells were suspended in PBS at 4 x 10⁷ cells /mL and mixed with equal volume of Matrigel (Corning, NY, U.S.A.) on ice (final 2 x 10⁷ cells /mL). 50 μ L (10⁶ cells) of the cell suspension was inoculated subcutaneously in hind legs of the mice. The mice were maintained as described above until tumors grew to 5-10 mm. Their body weight and tumor size were measured every day after the tumor became visible. Tumor volume (V) was calculated using the equation: V (mm³) = $0.523 \times a \times b^2$, where a and b are length (mm) and width (mm) of tumor, respectively. Statistical significance was analyzed by Student's t test.

Treatment with 5-ALA and hyperthermia (ALA-HT treatment)

For treatment of the cell culture, 0.1 mM 5-ALA hydrochloride was added at 37°C for 2 h in DMEM without FBS, and then the cells were grown in CO₂ incubator with the temperature set at 37°C or 42°C for 24 h in DMEM with 10% FBS. Cells were harvested by trypsinization and stained with 0.2% trypan blue (Life Technologies Japan, Tokyo, Japan), and the cell death rate was calculated as percentage of trypan blue-stained cells per total cells.

To the tumor-xenografted mice, aqueous solution of 5-ALA hydrochloride (at final dose of 300 mg/kg body weight) or equal volume of sterile water was orally administrated when their tumor size reached 3-4 mm in length. 3 h after the administration, their hind legs with tumor xenograft were immersed in water bath at 43°C for 20 min as described [24].

Combined treatment with ALA-HT and sorafenib (SFN)

For *in vitro* experiments, indicated final concentrations of SFN were added to the cell culture simultaneously in combination with the ALA-HT treatment described above.

For *in vivo* experiments, HepG2-xenografted BALB/c-nu mice were treated every 3rd day orally with SFN at 25 mg/kg b. w. Half of the SFN-treated mice were subjected to the ALA-HT treatment as described above next day, and this set of co-treatment was repeated three times with 3-day intervals.

Histopathological analysis of the tumor xenografts

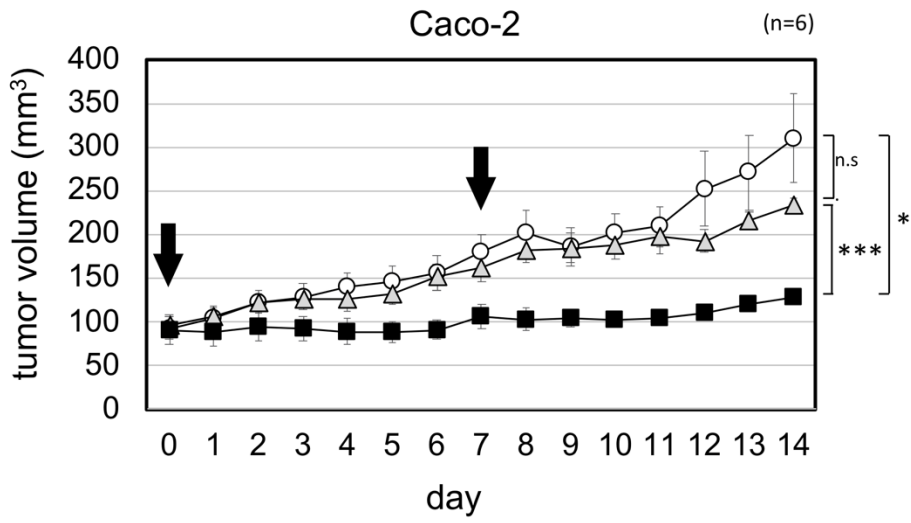
For histopathological analysis, tumors and surrounding normal tissues were excised when the tumors in the ALA-hyperthermia (ALA-HT) group were clearly smaller than those in the control and hyperthermia-only (HT) groups and showed no re-growth. The excised tumors were rinsed with PBS, cleaved with a scalpel, and shake-fixed with a 3.7% formaldehyde-PBS solution overnight at room temperature. Preparation of tissue sections and histopathological analysis based on hematoxylin-eosin (HE) staining were outsourced to Ina Research Inc, (Nagano, Japan).

Results

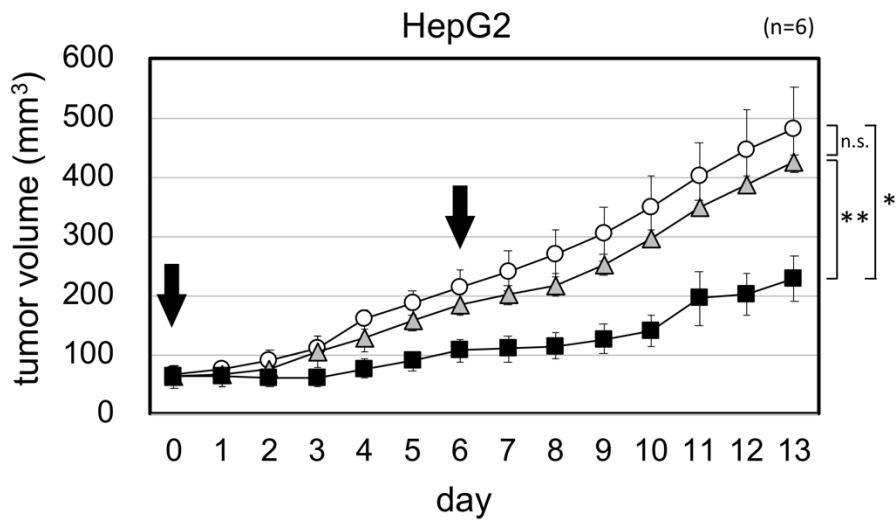
ALA-HT effect on tumor growth in mouse xenograft models

To examine the effect of co-treatment with 5-ALA and hyperthermia (ALA-HT) on tumor growth *in vivo*, two tumor-derived cell lines Caco-2 (colon adenocarcinoma) and HepG2 (liver cancer-derived) were xenografted into BALB/c-nu nude mice since these cell lines have been shown to be sensitive to the ALA-HT treatment *in vitro* [21]. The tumor-xenografted mice were subjected to hyperthermic treatment with or without oral administration of 5-ALA, with 6- or 7-day interval. As shown in Fig. 1, the growth of tumors derived from Caco-2 (A) or HepG2 (B) cells was significantly suppressed by ALA-HT treatment, compared to those treated with hyperthermia (HT) only. However, in both xenografts (especially in those derived from HepG2), ALA-HT treatment did not completely inhibit the tumor growth, resulting in regrowth of the tumors 3-4 days after the second ALA-HT treatment. We therefore repeated the experiments using HepG2-derived xenografts, with relatively smaller (younger) tumors at the start and shorter (3-day) intervals of treatments. As shown in Fig. 1 (C), although the ALA-HT treatment significantly suppressed the tumor growth compared to HT-only treatment, tumor regrowth was gradually observed again, especially after the fourth treatment. We also observed that HT-only treatment suppressed the HepG2-derived tumor growth to greater extent than in the previous results shown in Fig. 1 (B). We speculate this might be ascribed to the shorter intervals of the treatment since the HT-only treatment with 6- to 7-day intervals caused moderate suppressive effects lasting for a few days on the tumor growth both in HepG2 and Caco-2 xenografts (Fig. 1A).

A



B



C

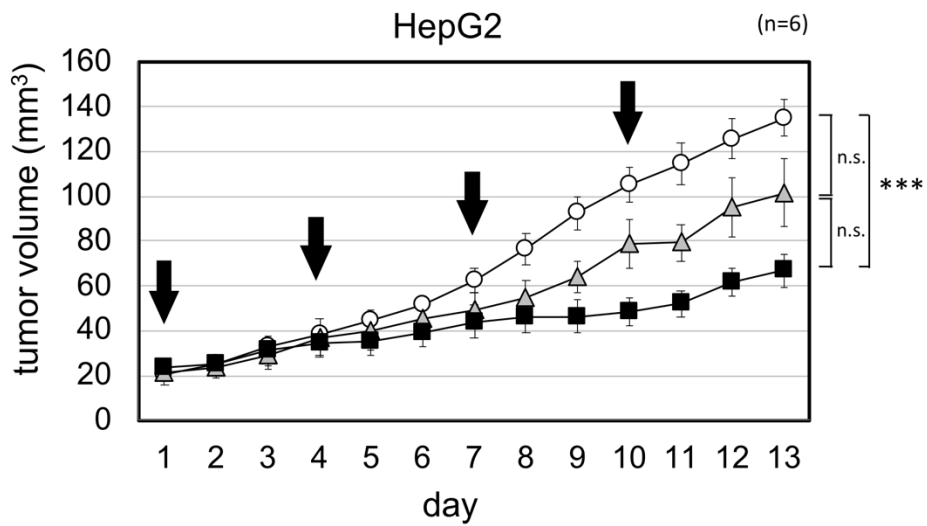
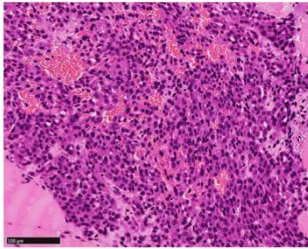


Fig. 1. Effect of 5-ALA and hyperthermic (HT) treatment on tumor growth in xenografted mice.

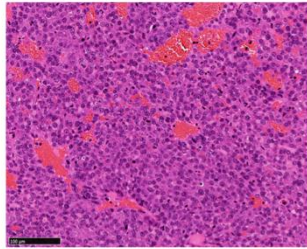
(A, B) BALB/c-nu mice bearing Caco-2 (A) or HepG2 (B) xenografts were orally given sterile water (control and HT) or 5-ALA at 300 mg/kg body weight (ALA-HT) on the days indicated by arrows (6- or 7-day interval). 3 h after the administration, HT and ALA-HT groups were subjected to hyperthermic treatment at 43°C for 20 min. Tumor volumes are plotted as mean \pm S.E. (n=6) for control (open circles), HT only (gray triangles), and ALA-HT (closed squares) treatment. Statistical significances between HT and ALA-HT and between control and ALA-HT groups are shown as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. There are no statistical significances between control and HT groups (n.s.). (C) HepG2-xenografted mice were treated as in (B), with younger (smaller) tumors and shorter (3-day) intervals of HT or ALA-HT treatment indicated by arrows. Tumor volumes are plotted as mean \pm S.E. (n=6) for control (open circles), HT only (gray triangles), and ALA-HT (closed squares) treatment. Statistical significance between control and ALA-HT groups is shown as *** $p < 0.001$. There are no statistical significances between control and HT, and between HT and ALA-HT groups (n.s.).

We then carried out histopathological analysis of those HepG2-derived tumors under the three conditions of treatment. Five independent tumors from the ALA-HT treatment group, clearly smaller than those from the control and HT-only treatment groups, as well as the same number of tumors from other two groups, were excised for tissue sections and subjected to histopathological analysis (Fig. 2). As summarized in Table 1, there is no obvious difference, in terms of the number and the grade of cell proliferation, necrosis, and inflammatory invasion observed, between the tumors from ALA-HT group and control or HT-only group.

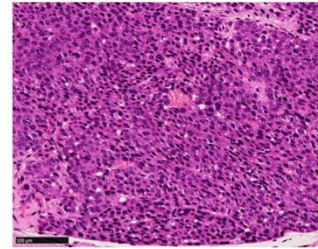
HepG2/control : ①-4L



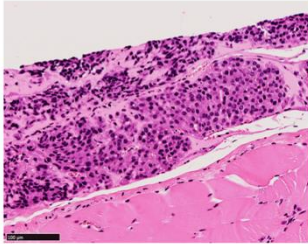
HepG2/HT : ②-3R



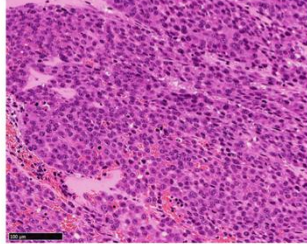
HepG2/ALA-HT : ⑤-2R



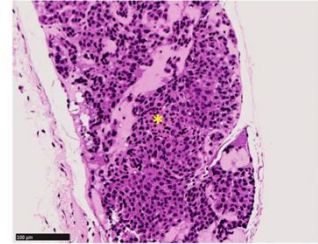
HepG2/control : ②-2R



HepG2/HT : ③-3L

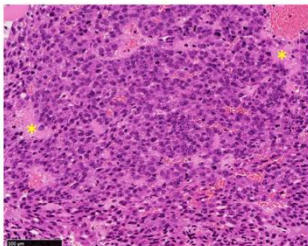


HepG2/ALA-HT : ⑤-3R



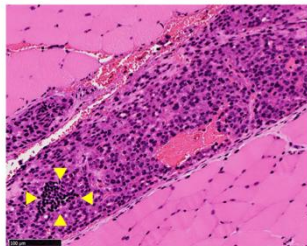
(*) focal necrosis grade 1

HepG2/control : ②-4L



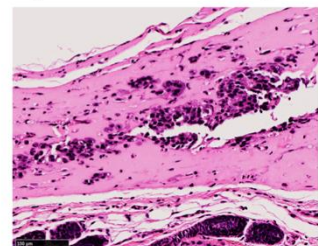
(*) focal necrosis grade 1

HepG2/HT : ③-4R

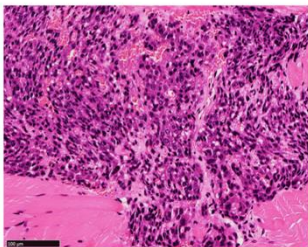


(▼) inflammatory cell infiltration grade 1 (mononuclear cells)

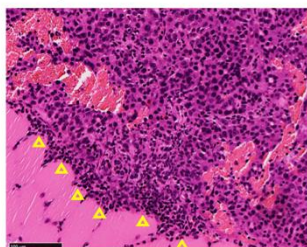
HepG2/ALA-HT : ⑥-3L



HepG2/control : ④-3L

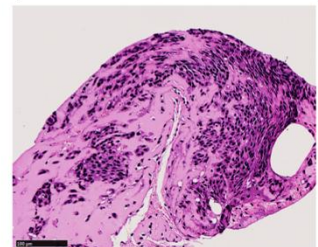


HepG2/HT : ④-2R

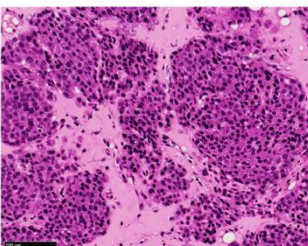


(Δ) inflammatory cell infiltration grade 1 (mononuclear cells/neutrophils)

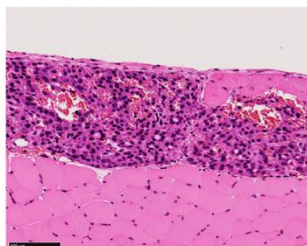
HepG2/ALA-HT : ⑥-3L(2)



HepG2/control : ⑥-4R



HepG2/HT : ⑥-1R



HepG2/ALA-HT : ⑦-3L

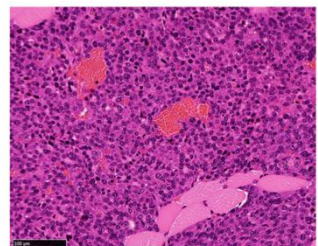


Fig. 2. Histopathological images of the HepG2-derived tumors.

Tissue sections were prepared from the tumors excised from the HepG2-xenografted mice with no treatment (control), hyperthermia treatment only (HT), and hyperthermia and 5-ALA co-treatment (ALA-HT), followed by HE staining. Scale bars: 100 μm . Asterisks: focal necrosis grade 1, closed triangle: inflammatory cell infiltration grade 1 (mononuclear cells), open triangles: inflammatory cell infiltration grade 1 (mononuclear cells/neutrophils).

Table 1 Histopathological analysis of HepG2-derived tumor sections.

Group	Section ID	Histopathological grading [†]			
		cell division	single cell necrosis	focal necrosis	inflammatory cell infiltration
HepG2/control	①-4L	1	1	0	0
	②-2R	0	1	0	0
	②-4L	1	1	1	0
	④-3L	0	1	0	0
	⑥-4R	1	1	0	0
HepG2/HT	②-3R	1	1	0	0
	③-3L	1	1	0	0
	③-4R	1	1	0	1a
	④-2R	1	1	0	1b
	⑥-1R	0	1	0	0
HepG2/ALA-HT	⑤-2R	1	1	0	0
	⑤-3R	1	1	1	0
	⑥-3L	0	1	0	0
	⑥-3L(2)	0	1	0	0
	⑦-3L	1	1	0	0

[†] 0: normal, 1: slight, 2: mild, 3: moderate, 4: severe

a : mononuclear cells, b : mononuclear cells/neutrophils

Based on these results, we conclude that, even though the ALA-HT treatment exerts a significantly suppressive effect on tumor growth *in vivo*, the effect under the conditions we employed is not sufficient for regression or elimination of tumors.

ALA-HT effect on the HepG2 cell death by a low-dose anti-tumor drug, sorafenib, in vitro

To overcome the limited effect of ALA-HT treatment on tumor growth as described above, we asked if the ALA-HT treatment can sensitize tumors to a low dose of anti-tumor drug. We employed sorafenib (SFN), a multi-kinase inhibitor that has been shown efficacy against a wide variety of tumors including liver cancer [25,26] and HepG2-derived tumor as a target drug-tumor model since HepG2 cells are derived from liver cancer. First, HepG2 cells were treated *in vitro* with various concentrations of SFN with or without ALA and/or HT treatment. Fig. 3 shows that the cell death rate of HepG2 increased in a dose-dependent manner when treated with 0.1 to 8 μM SFN. Combining SFN with ALA- or HT-only treatment enhanced the cell death 2- to 3-fold and co-treatment with SFN and ALA-HT resulted in about 4-fold enhancement, compared to those treated with equivalent concentrations of SFN only. Although the enhancement includes the effects of ALA and/or HT treatment without SFN, the SFN dose effect was enhanced ca. 80-fold at maximum with ALA-HT treatment since combination of 0.1 μM SFN and ALA-HT treatment resulted in the cell death rate (19.0%) almost equivalent to that with 8 μM SFN only (19.5%).

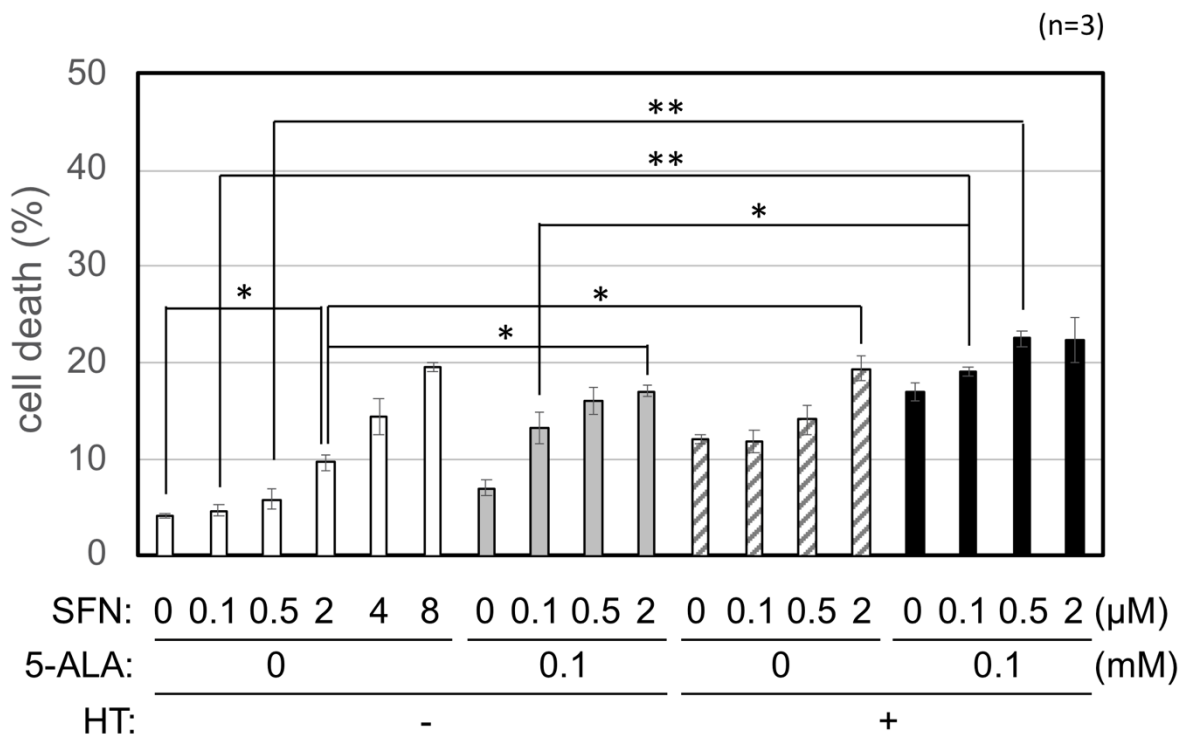


Fig. 3. Effect of combinations of sorafenib (SFN), 5-ALA and/or hyperthermic (HT) treatment on HepG2 cell growth *in vitro*.

HepG2 cells were grown with indicated concentrations of SFN and/or 5-ALA in the culture media at 37°C (HT-) or 42°C (HT+) for 24 h and subjected to trypan blue staining. Cell death rates were calculated as ratio (%) of trypan blue-stained cells per total cells and plotted as mean \pm S.E. (n=3) for SFN alone (open bars), SFN + 5-ALA (gray bars), SFN + HT (hatched bars), and SFN + ALA-HT (closed bars) treatment. Statistical significances are shown as: * $p < 0.05$, ** $p < 0.01$.

ALA-HT effect on the suppression of HepG2-derived tumor growth by a low-dose sorafenib in vivo

We then examined the effect of co-treatment with a low-dose SFN and ALA-HT on the growth of HepG2-derived tumors in mice. Each dose of SFN administrated was 25 mg/kg b. w., which is much lower (1/12 in total dose after three times) than a conventional SFN dose (100 mg/kg b. w. orally daily) in tumor-xenografted mice [27]. Half of the SFN-treated mice were subjected to ALA-HT treatment next day, and this set of co-treatment was repeated three times. As shown in Fig. 4, while treatment with the low-dose SFN did not significantly suppress the tumor growth, co-treatment with ALA-HT markedly and significantly enhanced the growth suppression by SFN. These results suggest that ALA-HT treatment can sensitize tumors to a low dose of SFN *in vivo*.

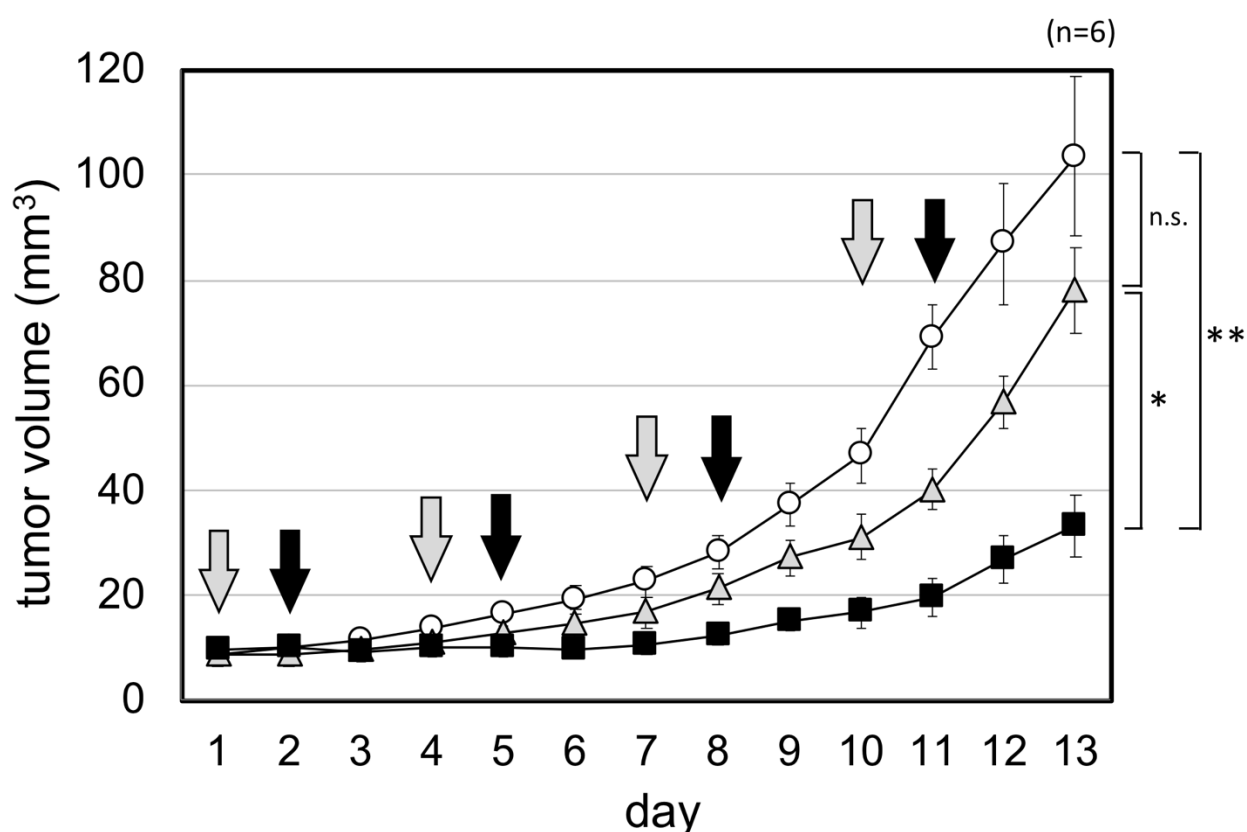


Fig. 4. Effect of co-treatment with SFN and ALA-HT on the growth of HepG2-derived tumors xenografted in mice.

HepG2-xenografted BALB/c-nu mice were administrated with vehicle (DMSO) or 25 mg/kg b. w. SFN on the days 1, 4, 7 and 10 (gray arrows), and the next day (closed arrows), half of the SFN-administrated groups were administrated with 300 mg/kg b. w. 5-ALA, and 3 h later, subjected to HT treatment at 43°C for 20 min. Tumor volumes were plotted as mean \pm S.E. (n=6) for control (open circles), SFN alone (gray triangles), and SFN + ALA-HT (closed squares). Statistical significances between SFN alone and SFN + ALA-HT, and between control and SFN + ALA-HT

are shown as: * $p < 0.05$, ** $p < 0.01$. There are no statistical significances between control and SFN alone groups (n.s.).

Discussion

ALA-HT treatment exerted stronger anti-proliferative effect on Caco-2 cells than on HepG2 in the mouse xenograft model (Fig. 1), which appears to reflect our previous observation that Caco-2 is more sensitive to ALA-HT treatment than HepG2 *in vitro* [21]. We have suggested that the difference in ALA-HT effect *in vitro* is partly attributed to the levels of PpIX accumulation under the ALA-HT treatment in these cells. The PpIX accumulation level could also contribute to the susceptibility of the tumors to ALA-HT treatment *in vivo* since the PpIX transporter ABCG2 has been shown to be down-regulated under the thermal stress in Caco-2 but not in HepG2 [21].

Although we observed a significantly repressive effect of ALA-HT treatment on the xenografted tumors derived from Caco-2 and HepG2 cells, the effect is not sufficient for complete growth suppression of these tumors. Consistently, the pathological analyses showed no evidence of cell proliferation arrest or cell death in the HepG2-derived tumors upon the ALA-HT treatment (Table 1 and Fig. 2). We should note, however, that some tissue sections shown in Fig. 2 reveal that xenografted tumors were not only subcutaneous but infiltrating into muscle, which could limit our interpretation. Nonetheless, those results from the pathological analyses are in contrast with our previous *in vitro* results indicating that ALA-HT treatment enhances the cell death in some cancer cell lines including Caco-2 and HepG2 [21]. We assume that the discrepancy is mainly derived from distinct conditions between *in vitro* and *in vivo* ALA-HT treatments. *In vitro*, the cells were treated with 0.5 to 2 mM of 5-ALA in the culture media at 42°C for 24 h [21], and *in vivo*, the tumor-xenografted mice were orally administrated with 300 mg/kg b. w. 5-ALA and the tumors were treated in 43°C for 20 min (this study). In rat models, the plasma 5-ALA concentration after the equivalent amount (300 mg/kg b. w.) of oral administration has been reported to be 0.3 mM [28]. Although the 5-ALA concentration was not measured in our mouse models, the *in vitro* 5-ALA concentration we have previously employed [21] appears to be much higher than the *in vivo* concentration in plasma, which could, at least in part, contribute to the cell death enhancement by ALA-HT treatment *in vitro* [21].

These observations prompted us to examine if ALA-HT treatment exhibits an enhancing effect on a low-dose anti-cancer drug, i.e. if ALA-HT treatment can sensitize tumor cells so as to reduce the drug's minimum dose. We chose sorafenib (SFN) as a test anti-cancer drug since it has been used for a broad range of cancers [25,26]. We observed a maximum 80-fold enhancement of SFN dose effect, i. e. co-treatment with 0.1 μM SFN and ALA-HT exhibited the cell death rate equivalent to that with 8 μM SFN only treatment in HepG2 cells (Fig. 3). Thus, SFN dose can be reduced down to 1/80 with an equivalent effect when combined with ALA-HT treatment *in vitro*. Then we examined the ALA-HT effect on low-dose SFN treatment of HepG2-derived tumors *in vivo*, which is numerically 1/12 of the conventional dose. This low dose of SFN exhibited only a

moderate, non-significant suppression of tumor growth, but significantly suppressed the growth of xenografted tumors when combined with ALA-HT treatment, compared to the treatment with SFN alone (Fig. 4). Therefore, similarly to our *in vitro* observation, we suggest that ALA-HT treatment can reduce the effective dose of SFN *in vivo*. We, however, also admit that our suggestion bears some limitation since we have not included a group of mice treated only with ALA-HT in the above experiment.

SFN has been reported to bear a potential risk of hemorrhagic and cardiac events [29,30] and also to cause moderate side effects such as diarrhea and hand-foot skin reaction in a significant population of patients in the large phase-3 clinical trial study [31]. Our findings suggest a possible clinical option employing ALA-HT as co-treatment for reducing the effective dose of SFN and thereby decreasing those side effects. This option could contribute to ameliorating the quality of life of patients undergoing cancer chemotherapy using not only SFN but also other anti-cancer drugs. Further evaluation of the effect of ALA-HT co-treatment with various anti-cancer drugs is expected to verify this possibility.

Conclusion

Our findings suggest that ALA-HT treatment can suppress tumor growth *in vivo* and also reduce the effective dosage of anti-cancer drug. This might contribute to amelioration of patients' quality of life in cancer chemotherapy.

Acknowledgments

Authors thank Drs. N. Higashi and K. Ohtsuka for providing cells, and F. Abe, A. Kamiya, Y. Toriyabe, M. Kawakami, H. Kuwamura, T. Sato, A. Haraguchi, M. Asanuma, M. Ishii, S. Ozawa, Y. Nakamura, K. Takemoto, Y. Onoue, and Y. Yokota for technical assistance. This work was supported by Tokyo University of Agriculture and in part by SBI Pharmaceuticals Co., Ltd.

References

- 1 G.A. Hendry, O.T. Jones, *J. Med. Genet.*, 1980, **17**, 1-14.
- 2 S. Ogura, K. Maruyama, Y. Hagiya, Y. Sugiyama, K. Tsuchiya, K. Takahashi, F. Abe, K. Tabata, I. Okura, M. Nakajima, T. Tanaka, *BMC Res. Notes*, 2011, **4**, 66.
- 3 J.P. Wang, H.J. Kim, Y.J. Chen, J.S. Yoo, J.H. Cho, D.K. Kang, Y. Hyun, I.H. Kim, *J. Anim. Sci.*, 2009, **87**, 3589-3595.
- 4 J.P. Wang, J.H. Lee, H.D. Jang, L. Yan, J.H. Cho, I.H. Kim, *J. Anim. Physiol. Anim. Nutr. (Berl.)*, 2011, **95**, 417-423.
- 5 Y. Morokuma, M. Yamazaki, T. Maeda, I. Yoshino, M. Ishizuka, T. Tanaka, Y. Ito, R. Tsuboi, *Int. J. Dermatol.*, 2008, **47**, 1298-1303.
- 6 P.R. Rehani, H. Iftikhar, M. Nakajima, T. Tanaka, Z. Jabbar, R.N. Rehani, *J. Diabetes Res.*, 2019, **2019**, 4267357.

- 7 F. Yamamoto, Y. Ohgari, N. Yamaki, S. Kitajima, O. Shimokawa, H. Matsui, S. Taketani, *Biochem. Biophys. Res. Commun.*, 2007, **353**, 541-546.
- 8 Y. Hagiya, Y. Endo, Y. Yonemura, K. Takahashi, M. Ishizuka, F. Abe, T. Tanaka, I. Okura, M. Nakajima, T. Ishikawa, S. Ogura, *Photodiagnosis Photodyn. Ther.*, 2012, **9**, 204-214.
- 9 J.C. Kennedy, R.H. Pottier, *J. Photochem. Photobiol. B* 1992, **14**, 275-292.
- 10 Q. Peng, T. Warloe, K. Berg, J. Moan, M. Kongshaug, K.E. Giercksky, J.M. Nesland, *Cancer*, 1997, **79**, 2282-2308.
- 11 W. Stummer, U. Pichlmeier, T. Meinel, O.D. Wiestler, F. Zanella, H.J. Reulen, A.-G.S. Group, *Lancet Oncol.*, 2006, **7**, 392-401.
- 12 D. Zaak, R. Sroka, W. Khoder, C. Adam, S. Tritschler, A. Karl, O. Reich, R. Knuechel, R. Baumgartner, D. Tilki, G. Popken, A. Hofstetter, C.G. Stief, *Urology*, 2008, **72**, 345-348.
- 13 W.E. Grant, C. Hopper, A.J. MacRobert, P.M. Speight, S.G. Bown, *Lancet*, 1993, **342**, 147-148.
- 14 M. Kriegmair, R. Baumgartner, W. Lumper, R. Waidelich, A. Hofstetter, *Br. J. Urol.*, 1996, **77**, 667-671.
- 15 P. Soergel, X. Wang, H. Stepp, H. Hertel, P. Hillemanns, *Lasers Surg. Med.*, 2008, **40**, 611-615.
- 16 J.C. Bai-Habelski, A. Ko, C. Ortland, M. Stocker, A. Ebeling, U. Reinhold, *Exp. Dermatol.*, 2022, **31**, 1385-1391.
- 17 P. Agostinis, K. Berg, K.A. Cengel, T.H. Foster, A.W. Girotti, S.O. Golinick, S.M. Hahn, M.R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mros, D. Nowis, J. Piette, B.C. Wilson and J. Golab, *CA Cancer J. Clin.*, 2011, **61**, 250-281.
- 18 J. van der Zee, D. González González, G.C. van Rhoon, J.D. van Dijk, W.L. van Putten, A.A. Hart, *Lancet*, 2000, **355**, 1119-1125.
- 19 M. Urano, M. Kuroda, Y. Nishimura, *Int. J. Hyperthermia*, 1999, **15**, 79-107.
- 20 M.W. Dewhirst, E.J. Ozimek, J. Gross, T.C. Cetas, *Radiology*, 1980, **137**, 811-817.
- 21 T. Chibazakura, Y. Toriyabe, H. Fujii, K. Takahashi, M. Kawakami, H. Kuwamura, H. Haga, S. Ogura, F. Abe, M. Nakajima, H. Yoshikawa, T. Tanaka, *Biosci. Biotechnol. Biochem.*, 2015, **79**, 422-431.
- 22 J. Furuse, H. Ishii, K. Nakachi, E. Suzuki, S. Shimizu, K. Nakajima, *Cancer Sci.*, 2008, **99**, 159-165.
- 23 Y. Li, Z.H. Gao, X.J. Qu, *Basic Clin. Pharmacol. Toxicol.*, 2015, **116**, 216-221.
- 24 K. Takahashi, T. Hasegawa, T. Ishii, A. Suzuki, M. Nakajima, K. Uno, I. Yasuda, A. Kishi, K. Sadamoto, F. Abe, T. Tanaka, *Anticancer Res*, 2013, **33**, 2861-2866.
- 25 S.M. Wilhelm, C. Carter, L. Tang, D. Wilkie, A. McNabola, H. Rong, C. Chen, X. Zhang, P. Vincent, M. McHugh, Y. Cao, J. Shujath, S. Gawlak, D. Eveleigh, B. Rowley, L. Liu, L. Adnane, M. Lynch, D. Auclair, I. Taylor, R. Gedrich, A. Voznesensky, B. Riedl, L.E. Post, G. Bollag, P.A. Trail, *Cancer Res.*, 2004, **64**, 7099-7109.

- 26 L. Liu, Y. Cao, C. Chen, X. Zhang, A. McNabola, D. Wilkie, S. Wilhelm, M. Lynch, C. Carter, *Cancer Res.*, 2006, **66**, 11851-11858.
- 27 H. Huynh, V.C. Ngo, H.N. Koong, D. Poon, S.P. Choo, C.H. Thng, P. Chow, H.S. Ong, A. Chung, K.C. Soo, *J. Cell. Mol. Med.*, 2009, **13**, 2673-2683.
- 28 T. Owari, T. Iwamoto, S. Anai, M. Miyake, Y. Nakai, S. Hori, T. Hara, T. Ishii, U. Ota, K. Torimoto, H. Kuniyasu, T. Fujii, N. Tanaka, K. Fujimoto, *Photodiagnosis Photodyn. Ther.*, 2021, **34**, 102309.
- 29 D. Semela, J.F. Dufour, *J. Hepatol.*, 2004, **41**, 864-880.
- 30 L.S. Wood, B. Manchen, *Clin. J. Oncol. Nurs.*, 2007, **11**, 649-656.
- 31 J.M. Llovet, S. Ricci, V. Mazzaferro, P. Hilgard, E. Gane, J.F. Blanc, A.C. de Oliveira, A. Santoro, J.L. Raoul, A. Forner, M. Schwartz, C. Porta, S. Zeuzem, L. Bolondi, T.F. Greten, P.R. Galle, J.F. Seitz, I. Borbath, D. Häussinger, T. Giannaris, M. Shan, M. Moscovici, D. Voliotis, J. Bruix, S.I.S. Group, *N. Engl. J. Med.*, 2008, **359**, 378-390.