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Evaluation of the Sonosensitizing Activities of 5-Aminolevulinic Acid and Sn(IV) Chlorin e6 in Tumor-bearing Chick Embryos

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Abstract. *Background: Recently, 5-aminolevulinic acid (5-ALA), precursors of protoporphyrin IX (PpIX), and Sn(IV) chlorin e6 (SnCe6) have been proposed as possible sonosensitizers for sonodynamic therapy of cancer. Therefore, we evaluated the pharmacokinetic properties and sonosensitizing activities of 5-ALA and SnCe6 in vivo by using the EMT6/KU tumor-bearing chick embryos. Results: The concentration of PpIX in tumor and liver tissues and serum increased in a time-dependent manner after the i.v. administration of 5-ALA; PpIX concentrations reached their peak level after 5-7 h. The concentration of SnCe6 reached its maximum value in the tumor tissue and serum immediately after i.v. administration. The combined treatment of 5-ALA or SnCe6 with ultrasound irradiation showed a significant antitumor effect towards EMT6/KU solid tumors. Conclusion: We evaluated the pharmacokinetic properties and sonosensitizing activities of 5-ALA and SnCe6 in a chick embryo model and found that 5-ALA might be more suitable as a sonosensitizer than SnCe6.*

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Key Words: Pharmacokinetics, sonosensitizer, 5-aminolevulinic acid, Sn(IV) chlorin e6, tumor-bearing chick embryo.

Sonodynamic therapy (SDT) for cancer, which relies on the synergistic effects of ultrasound irradiation and sonosensitizers, was reported 20 years ago (1-3); however, the therapeutic potential of SDT remains unknown and it is poorly recognized as a cancer treatment. In SDT, photosensitizers such as porphyrins, chlorophylls, and phthalocyanines, have been reportedly used as sonosensitizers to generate reactive oxygen species in response to ultrasound irradiation (4-6). 5-Aminolevulinic acid (5-ALA), a precursor of protoporphyrin IX (PpIX), is currently used for photodynamic diagnosis and photodynamic therapy of several types of cancer (7, 8). Sn(IV) chlorin e6 (SnCe6) is a stannum-modified chlorophyll derivative and its tumor-targeting derivatives have been developed as photosensitizers (9, 10). 5-ALA and SnCe6 were recently proposed as sonosensitizers for SDT (11-13). However, no comparative studies have been carried out to evaluate the sonosensitizing activity of these compounds under the same experimental conditions when combined with ultrasound. High intensity focused ultrasound (HIFU) has been used as a highly effective therapy for prostate cancer (14). The therapeutic activity of HIFU is primarily attributed to the hyperthermic effect of the ultrasound energy, which can deeply penetrate cancer cells. SDT using ultrasound and sonosensitizers was developed based on the principle that non-thermal ultrasound energy can induce the cytocidal actions of reactive oxygen species. However, no conventional *in vivo* assay systems exist to

evaluate the activity of putative sonosensitizers in SDT. Here, we report the use of the tumor-bearing chick embryo as an animal model to investigate the pharmacokinetic properties and sonosensitizing activities of 5-ALA and SnCe6.

Materials and Methods

Chemicals. 5-ALA hydrochloride was a gift from SBI Pharmaceuticals Co., Ltd. (Tokyo, Japan). SnCe6 was synthesized in our laboratory according to a patented method (15) by using chlorin e6 (Funakoshi Co., Ltd., Tokyo, Japan) as a starting material. Triton X-100 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Meylon was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cell culture. Mouse mammary EMT6/KU tumor cells (supplied by Dr. Shin-ichiro Masunaga, Kyoto University, Kyoto, Japan) were maintained in Eagle's minimum essential medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (JR Scientific, Inc., Woodland, CA, USA). Cells were cultured in a 5% CO_2 in a fully-humidified atmosphere at 37˚C.

Inoculation of EMT6/KU cells onto chorioallantoic membrane. Fertilized chicken eggs were purchased from Goto Hatchery, Inc. (Gifu, Japan). The first day of incubation was considered day 1. The fertilized chicken eggs were incubated in a humidified incubator at 37.6˚C until day 11. On day 11, eggs were candled using halogen light to mark Y-shaped blood vessels on the chorioallantoic membranes (CAM). Over each of the identified blood vessels, a 1.5×1.5 cm square window was cut into the eggshell by using a grinder. After the eggshell and membrane were removed, the windows were sealed with polyurethane film (Opsite, Smith & Nephew, London, UK). Teflon rings were placed on the Y-shaped blood vessels and 2.5×10^5 EMT6/KU cells/embryo were transferred to the Teflon ring. The window was resealed with transparent polyurethane film (3M Tegaderm™, St. Paul, MN) and the eggs were incubated at 37.6˚C for 48 h (16). The Teflon rings were removed on day 13 and the eggs were incubated until day 15.

Intravenous administration of sonosensitizers in tumor-bearing chick embryos. On day 15, the eggs prepared with the above section were candled using halogen light to mark a thin blood vessel on the CAM. Rectangular 0.5×2 cm windows were cut into the eggshells over the thickest blood vessels. To visualize the blood vessels under the membrane, liquid paraffin was dropped onto the eggshell membrane. 5-ALA and SnCe6 were dissolved in physiological saline and meylon, respectively. Intravenous (*i.v.*) administration of 0.1 ml of each sensitizer was performed using a 30-gauge needle (16). After the *i.v.* administration, tumor, liver, and blood samples were collected at intervals up to 24 h. Serum was prepared from blood by using centrifugation at 3,000 rpm (735 × *g*) for 10 min. Tumors and livers were rinsed twice in physiological saline and stored at −80˚C until they were analyzed.

Analysis of sonosensitizers. Tumor and liver tissues stored at −80˚C were thawed at room temperature, weighed, and then homogenized in a solution of 2% Triton X-100 using a Handy micro-homogenizer Physcotron (Microtec Co., Ltd., Chiba, Japan) at 24,000 rpm for 10 s. After homogenization, the sample solutions were centrifuged at 15,000 rpm (160,000 × *g*) for 15 min and their supernatants were collected in new microcentrifuge tubes. PpIX and SnCe6 in the supernatants were quantified by measuring the fluorescence intensity using a LLS-405 VIS LED light source for excitation and a SEC2000-VIS/NIR fluorescence detector (BAS Inc., Tokyo, Japan). The excitation and emission wavelengths of PpIX were 400 nm and 632 nm, respectively, whereas the excitation and emission wavelengths of SnCe6 were 400 nm and 641 nm, respectively. The concentrations of PpIX and SnCe6 in the samples were determined from standard curves.

In vivo ultrasound irradiation. On day 15, tumor-bearing chick embryos received ultrasound irradiation 10 min and 5 h after SnCe6 and 5-ALA administration, respectively. Irradiation was performed directly on the solid tumors on the CAM by using a Sonitron GTS equipped a 12 mm transducer (Nepagene Co., Ltd., Chiba, Japan) under non-thermal conditions (986 kHz, 2.5 W, 60% pulse, 10 min). On day 18, the chick embryos were sacrificed and tumors were dissected from the CAMs.

Statistical analysis. Data are expressed as the mean±standard deviation. The differences between the results of the independent experiments were statistically analyzed using Student's *t*-test. A *p*-value <0.05 was considered statistically significant. Thompson's rejection tests were performed for individual values.

Results

Pharmacokinetics of sonosensitizers in tumor-bearing chick embryos. After the *i.v.* administration of 5-ALA, the concentration of PpIX in the tumor tissue, liver tissue, and serum increased in a time-dependent manner, reaching its maximum concentration after 5 to 7 h (Figure 1). The concentration of PpIX in all tissue samples decreased markedly at 24 h after 5-ALA administration. SnCe6 in the tumor and serum samples reached its peak concentration immediately (1 min) after it was administered by *i.v.* (Figure 2A and C). The serum concentration rapidly decreased and SnCe6 became undetectable 24 h later. The concentration of SeCe6 in the tumors slowly decreased during the 24-h incubation period. In contrast, the concentration of SeCe6 in the liver tissue gradually increased after it was administered *i.v.*, reaching its maximum concentration within 5 h (Figure 2B).

In vivo sonosensitizing activity of 5-ALA and SnCe6. Administration of 1.0 mg of 5-ALA followed by 5 h of ultrasound irradiation was performed to evaluate the antitumor effects of the combined treatment. Ultrasound irradiation was performed 5 h after 5-ALA administration when the PpIX concentration had reached its peak in the tumors. The tumor weights in the control (physiological saline) single and combined treatment groups are shown in Figure 3A. The tumor growth suppression rates were 14% for 5-ALA alone and 56% for the combined treatment, whereas tumor growth was not suppressed by ultrasound alone. The combined treatment of ultrasound and 5-ALA suppressed tumor growth to a greater extent than did the

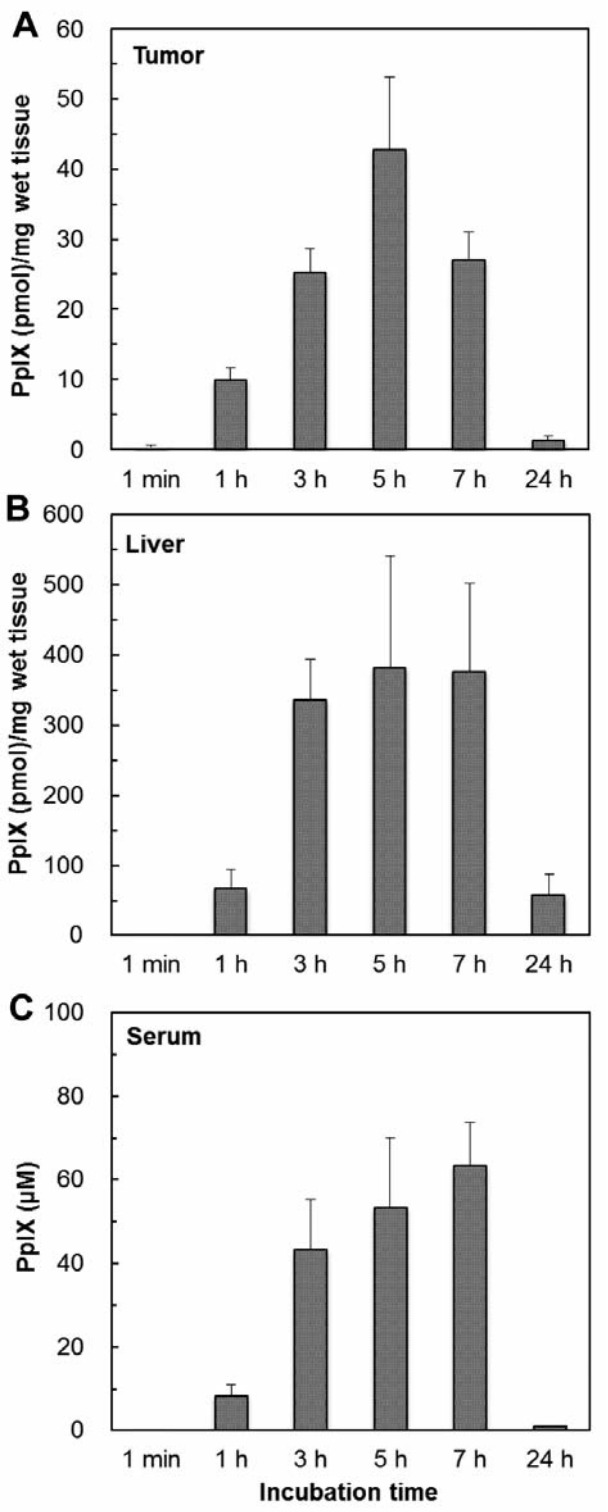

Figure 1. *Time-dependent change in protoporphyrin IX (PpIX) concentration in tumor-bearing chicken embryos after 5-aminolevulinic acid (5-ALA) administration. Concentration of PpIX in tumor (A) and liver (B) tissues, and in serum (C). At each indicated time point, 4-5 eggs were evaluated. Values are the mean±standard deviation (SD). Error bars represent the standard deviation.*

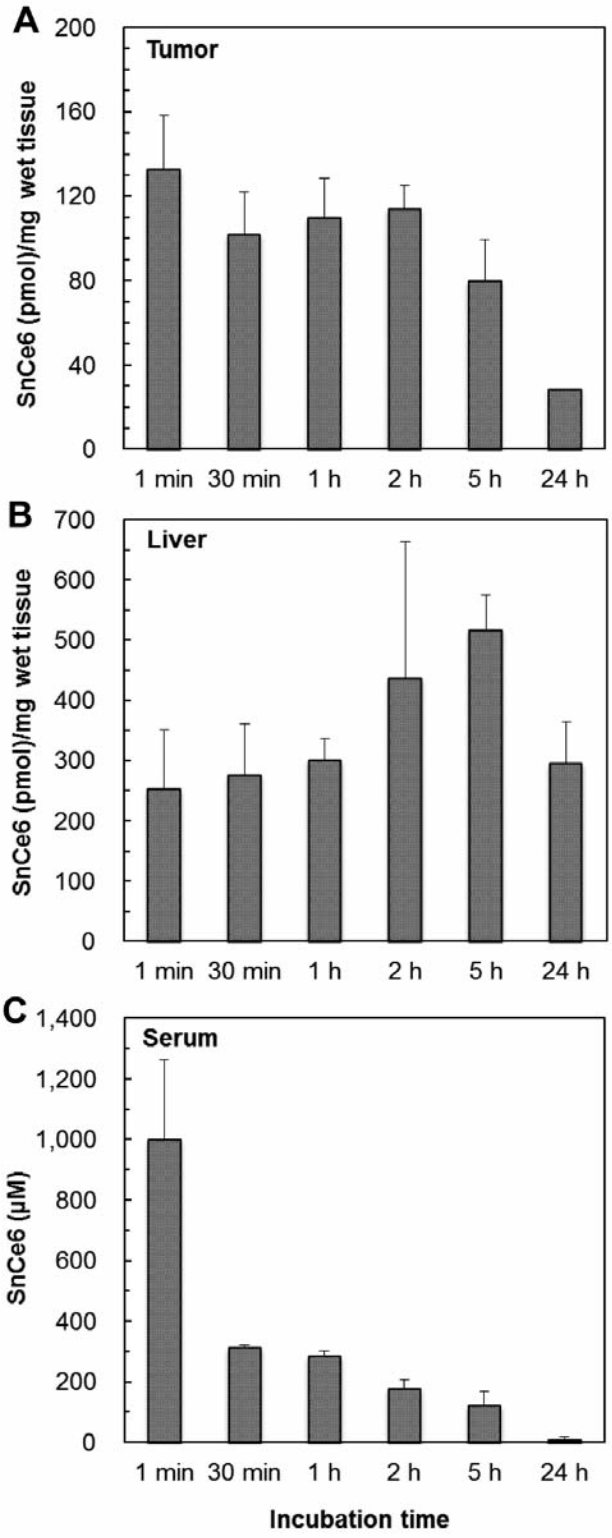

Figure 2. *Time-dependent change in Sn(IV) chlorin e6 (SnCe6) in tumor-bearing chick embryos. Concentration of SnCe6 in tumor (A) and liver (B) tissues, and in serum (C). At each indicated time point, three eggs were evaluated. Values are the mean±standard deviation (SD). Error bars represent the standard deviation.*

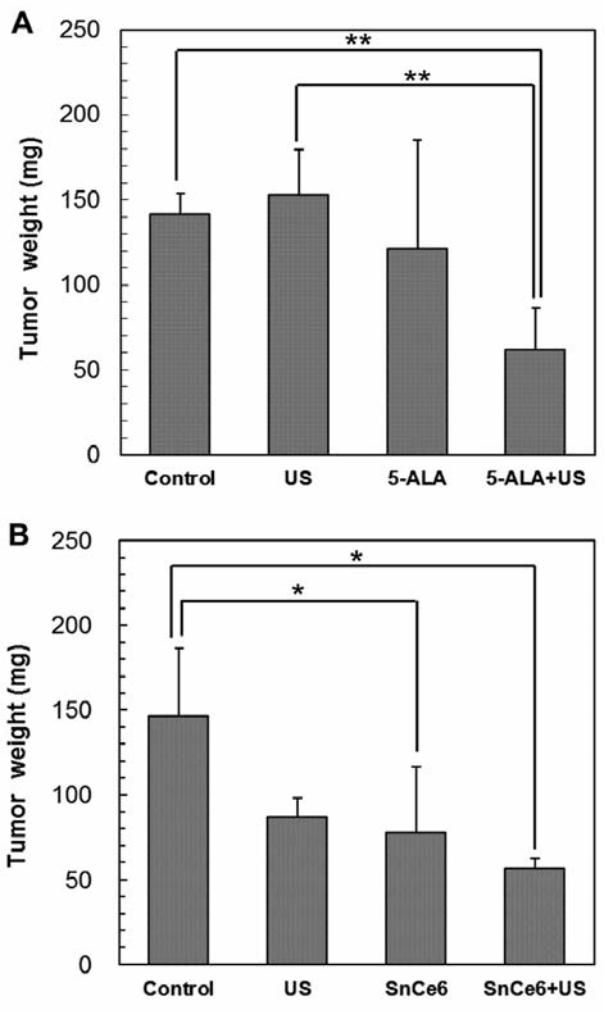

Figure 3. *The sonosensitizing activity of 5-aminolevulinic acid (5-ALA) (A) and SnCe6 (B) in tumor-bearing chick embryos. The numbers of surviving/used eggs were as follows: A: Control, 4/6; ultrasound (US) alone, 3/5; 1.0 mg of 5-ALA, 3/4; and combined treatment of ultrasound and 1.0 mg of 5-ALA, 5/5; B: control, 4/5; ultrasound alone, 3/3; 1.0 mg of SnCe6, 4/4; and combined treatment of ultrasound and 1.0 mg of SnCe6, 3/3. One egg from each of the ultrasound-alone and combined-treatment groups were rejected using the Thompson's rejection test. Values are the mean±standard deviation (SD). Error bars represent the standard deviation. *p<0.05 and **p<0.01.*

control treatment and ultrasound irradiation alone ($p<0.01$). The difference between 5-ALA alone and the combined 5-ALA/ultrasound treatment was not significant because of the large SD obtained for the 5-ALA-alone group; however, the mean tumor weight in the combined treatment group was approximately half of that of the group treated with 5-ALA alone ($p=0.0504$).

Next, we evaluated the antitumor effect of the combined treatment of 1.0 mg SnCe6 and ultrasound irradiation. Based on the pharmacokinetic data of SnCe6 in the chick embryo, the ultrasound irradiation was performed 10 min after SnCe6 administration. The tumor weights in the different groups are shown in Figure 3B. The tumor growth-suppression rates in the ultrasound irradiation-alone, SnCe6-alone, and combined SnCe6/ultrasound treatment groups were 41%, 47%, and 61%, respectively. Significant tumor growth suppression was observed in the group with SnCe6-alone and the combined-treatment group compared to the control group ($p<0.05$).

Discussion

In the present study, we aimed to evaluate the pharmacokinetic properties and sonosensitizing activities of 5-ALA and SnCe6 *in vivo* by using the tumor-bearing chick embryo as an animal model. The intratumoral distribution of 5-ALA in the chick embryos were comparable to that observed in other animal models (17, 18). The maximum intra-tumoral concentration of PpIX in the chick embryos was observed 5 h after 5-ALA was administered using *i.v.*, while the peak concentration in pancreatic tumors transplanted into golden hamsters and human colon tumors transplanted into hairless mice were observed after 4 h (17) and 6.2 h (18), respectively. While the concentration of PpIX in the livers of mice reached its maximum after 1 h and dissipated within 5 h (18), PpIX in the livers of chicken embryos reached its maximum concentration 5-7 h after 5-ALA administration and was detected at 10 times the level in tumor. We attributed these differences in PpIX pharmacokinetics after 5-ALA administration to the transport of PpIX gradually by tumor cells and livers into the blood or due to a slower metabolic rate of 5-ALA and PpIX in the livers of the chicken embryos compared to that in the livers of mice. Pharmacokinetic analysis of SnCe6 has not been performed in other systems, but our data are in agreement with the results of a study on the pharmacokinetics of chlorin e6, a non-metal analog of SnCe6 (19).

With respect to the *in vivo* sonosensitizing activity of 5-ALA, Ohmura *et al*. reported that focused ultrasound (10 W/cm^2 at 1.04 MHz for 5 min) and 5-ALA (100 mg/kg) treatment had a significant antitumor effect *in vivo* in a rat model of deep-seated intracranial glioma (20). Our ultrasound conditions (3.4 W/cm^2 at 986 kHz for 10 min) were comparatively weak, yet our experimental data regarding the sonosensitizing effect 5-ALA was comparable to the results reported by Ohmura *et al*. (20). We speculate that tumors growing in chick embryos are more susceptible to SDT than those in mice.

In conclusion, we evaluated the pharmacokinetic properties and sonosensitizing activities of 5-ALA and SnCe6 in a chick embryo model and found that 5-ALA might be more suitable as a sonosensitizer than SnCe6.

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