Sonodynamically Induced Antitumor Effect of 4-Formyloximethylidene-3-hydroxy-2-vinyl-deuterio-porphynyl(IX)-6,7-diaspartic Acid (ATX-S10)

Nagahiko Yumita,[1] Ryuichiro Nishigaki,[1] Isao Sakata,[2] Susumu Nakajima[3] and Shin-ichiro Umemura[4, 5]

[1]School of Pharmaceutical Sciences, Toho University, Miyama, Funabashi, Chiba 274-8510, [2]Photochemical Co., Ltd., Haga, Okayama, Okayama 701-1221, [3]Division of Surgical Operation, Asahikawa Medical College, Nishi-Kagura, Asahikawa, Hokkaido 078-8307 and [4]Central Research Laboratory, Hitachi Ltd., Higashi-Koigakubo, Kokubunji, Tokyo 185-8601

The sonodynamically induced antitumor effect of 4-formyloximethylidene-3-hydroxy-2-vinyl-deuterio-porphynyl(IX)-6,7-diaspartic acid (ATX-S10) was investigated. Both *in vitro* and *in vivo* antitumor effects were tested in combination with ultrasound at 2 MHz. The rate of ultrasonically induced damage to isolated sarcoma 180 cells in air-saturated suspension was enhanced two-fold with 80 μM ATX-S10. This enhancement was significantly inhibited by histidine, which may suggest that it was mediated by ultrasonically induced oxidation. The coadministraion of 25 mg/kg ATX-S10 followed by ultrasonic exposure at 2 MHz stopped the growth of implanted colon 26 tumors at an intensity at which ultrasound alone showed only a slight antitumor effect.

Key words: ATX-S10 — Antitumor effect — Sonodynamic therapy — Sarcoma 180 — Colon 26

Ultrasound has an appropriate tissue attenuation coefficient for penetrating intervening tissues to reach non-superficial objects while maintaining the ability to focus energy into small volumes. This is a unique advantage when compared to electromagnetic modalities such as laser beams, for application to non-invasive treatment of non-superficial tumors. Although the use of ultrasound for tumor treatment has been relatively well investigated with respect to the thermal effects due to ultrasound absorption, only a few studies have been reported with respect to non-thermal effects such as the sonochemical effects due to ultrasound cavitation.[1–3]

Recently we found that photochemically active porphyrins such as hematoporphyrin (Hp), a hematoporphyrin derivative (HpD),[4, 5] and a gallium porphyrin complex, 7,12-bis(1-decyloxyethyl)-Ga(III)-3,8,13,17-tetramethylporphyrin-2,18-dipropionyl diaspartic acid (ATX-70) also induce significant cell damage when activated with ultrasound.[6, 7] Ultrasonically induced cytotoxicity of certain porphyrins was also confirmed by other groups.[8, 9] Implanted mouse tumors were treated with ultrasound in a standing wave mode after administration of such porphyrins and the tumor growth was significantly inhibited at an intensity at which ultrasound alone showed only a slight inhibitory effect.[10] These results demonstrated that such porphyrins have potential for use as a sonochemical sensitizer for tumor treatment in combination with ultrasound, which may be referred to as "sonodynamic therapy." [11, 12] The effect of active oxygen scavengers on the ultrasonically induced cell damage and also the effect of deuterium

oxide substitution of hydrogen oxide in the suspension medium both suggest that singlet oxygen generated by sonochemically activated porphyrins is likely to be the most important cell-damaging mediator.[6, 7] Active oxygen generation in aqueous solution by ultrasonically activated porphyrins was confirmed by detecting the formation of 2,2,6,6-tetramethyl-4-piperidone-N-oxyl from 2,2,6,6-tetramethyl-4-piperidone by electron spin resonance spectroscopy.[6, 13]

A chlorin derivative, 4-formyloximethylidene-3-hydroxy-2-vinyl-deuterio-porphynyl(IX)-6,7-diaspartic acid, referred to as ATX-S10, whose chemical structure is shown in Fig. 1, showed a much longer phosphorescence lifetime than Hp or HpD. This long phosphorescence lifetime can be a great advantage in the efficient photochemical generation of singlet oxygen.[14] It has been reported that ATX-S10 showed significantly lower toxicity than ATX-70. The lethal dose of ATX-S10 to a mouse was an order of magnitude higher than that of ATX-70. Furthermore, like some porphyrins, ATX-S10 is more preferentially retained by tumors than normal tissues. Nakajima *et al.* studied ATX-S10 accumulation in colon 26 tumor tissue after intravenous injection. The highest concentration of ATX-S10 in tumor tissue was observed 6 h after the administration and the ratio of concentration between the tumor and other normal tissues reached about five. Significant tumor tissue destruction was demonstrated using ATX-S10 in combination with pulsed laser irradiation. These results suggest that ATX-S10 has great potential as a photosensitizer for photodynamic therapy.[14–16]

It would not be unnatural to expect that ATX-S10 can also be activated by ultrasound, like the above described porphyrins, and that its use in combination with ultrasonic exposure may also be effective for tumor treatment. In this
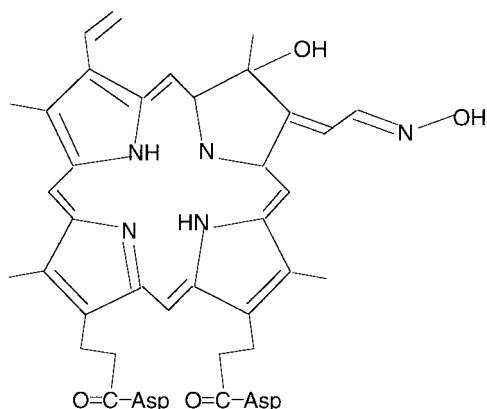
Fig. 1.   Chemical structure of ATX-S10.

paper, sonodynamically induced *in vitro* and *in vivo* effects of ATX-S10 were investigated on experimental tumors using ultrasound at 2 MHz in standing wave modes.

MATERIALS AND METHODS

Chemicals ATX-S10 was supplied by Toyo Hakka Kogyo (Okayama). Histidine, mannitol, and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were commercial products of analytical grade.

Evaluation of *in vitro* effect Sarcoma 180 cells was supplied by Meiji Seika Kaisha (Tokyo). The cell lines were passaged weekly through male ICR mice in ascites form. Cells were harvested from the peritoneal cavity of a tumor-bearing animal 7 to 10 days after inoculation. The tumor cells harvested from mice were suspended in an air-saturated phosphate buffer solution (PBS, pH 7.4) and were packed by light centrifugation ($100g$, 1 min). Then the cells were resuspended in PBS at a concentration of 4×10^6 cells/ml. The cell suspensions were stored on ice until used in the experiments.

The viability of the isolated cells was determined by staining of the cells with Trypan Blue dye. A 1 ml aliquot was taken from the cell suspension and mixed with 1 ml of 0.5% Trypan Blue solution. The integrity of the cells was determined by counting the number of unstained cells on a hemocytometer glass plate using an optical microscope. It was checked before every treatment, and cell suspensions with above 99% integrity were used in a series of experiments. This number of intact cells before treatment was regarded as the standard for the integrity determination after insonation.

A 2.5 ml portion of the cell suspension was transferred to an exposure chamber and insonated. The extent of ultrasonically induced cell damage in the presence and absence of 80 μM ATX-S10 in suspensions with and without potential active oxygen scavengers was determined by comparing the integrity before and immediately after insonation. Each result is presented as the mean with standard deviation (SD) of four insonation experiments.

Evaluation of antitumor effect Colon 26 carcinoma was supplied by the Cancer Institute (Tokyo). The cell lines were passaged weekly through male BALB/c mice (5 weeks old). Transplanted tumors were initiated by subcutaneous trocar injection of approximately 1 mm³ pieces of fresh tumor into the left dorsal scapula region of male 5-week-old CDF₁ mice. When the tumor grew to a diameter of about 10 mm approximately 14 days after implantation, the treatment study was started. The tumor-bearing mice were divided into four groups of four mice: (1) the control group, and those treated with (2) ATX-S10, (3) ultrasound, or (4) ATX-S10+ultrasound. For the treatments with ATX-S10, ATX-S10 was administered to mice from the caudal vein at the dose of 25 mg/kg. This dose of ATX-S10 was used by Nakajima *et al*. to study accumulation in tumor tissues.[14, 15] They reported that a high tumor-to-normal-tissue concentration ratio was observed within 6 h after the administration. For the combined treatment, 6 h after the administration was chosen for the insonation timing based on their results.

The long and short diameters (a and b in mm) of the tumor were measured with a slide caliper every 7 days after inoculation. The tumor size was calculated as $(a+b)/2$. The mean and SD were calculated for each group consisting of four mice. The values were compared by the use of Student's t test with $P<0.05$ as the criterion of signifi-
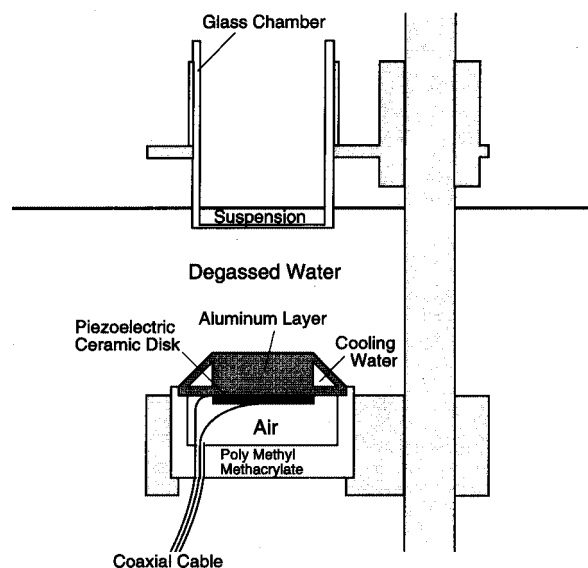

Fig. 2.   Cross-sectional view of *in vitro* insonation set-up.

cance. Four weeks after the treatment, the mice were killed and the tumors were dissected out and weighed.

***In vitro* insonation** The *in vitro* exposure set-up is shown in Fig. 2. The air-backed transducer used a lead-titanate piezoelectric ceramic disk of 24 mm diameter, purchased from Hitachi Metals (Tokyo), and was tightly bonded onto an aluminum layer with a low heat-expansion epoxy adhesive. The overall resonant frequency of the transducer was 1.92 MHz. Sine waves were generated by a wave generator (model MG442A, Anritsu Electric, Tokyo) and amplified by an RF amplifier (model 210L, ENI, Rochester, NY). The sinusoidal drive signal of the transducer was monitored with an oscilloscope during the ultrasonic exposure.

The transducer was submerged in degassed water at room temperature facing upward, with its acoustic surface parallel to the water surface. An insonation glass container of 31 mm diameter with a flat bottom layer of 1.5 mm thickness was placed 30 mm from the transducer. A 2.5 ml aliquot of air-saturated suspension was placed in the container. The level of the degassed water was approximately adjusted to the level of the suspension or solution in the container. The ultrasound attenuation through the bottom layer of the container for insonation was also estimated in a propagation mode, using a needle-type hydrophone by comparing acoustic pressure on the axis with and without the layer between the transducer and the hydrophone. When the layer was parallel to the transducer surface, the attenuation was less than 10% in amplitude. It may have been small because the thickness of the layer was close to a half wavelength and the acoustic field was close enough to a plane wave field. The temperature rise in 2.5 ml of air-saturated water in the glass container during the insonation was checked using a 0.25 mm diameter Chromel-Almel thermocouple. It was less than 1°C for 1 min insonation at the free field intensity of 4.5 W/cm².

***In vivo* insonation** The *in vivo* ultrasonic exposure set-up is shown in Fig. 3. The air-backed transducer used a lead-

zirconate-titanate ceramic disk of 12 mm diameter, purchased from Fuji Ceramics (Fujinomiya, Shizuoka), and was tightly bonded onto an aluminum layer, which was cooled by circulating water to keep the transducer and tumor temperature below a certain level. The overall resonant frequency of the transducer was 2.0 MHz. A tumor-bearing mouse a week after inoculation was anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The hair over the tumor was shaved and ultrasound gel was applied to the naked skin. The mouse was fixed on a cork board and the transducer was placed tightly on the tumor. The tumor was insonated in a standing wave mode at the free-field intensity of 3 W/cm² for 15 min. The transducer was cooled by circulating water at 25°C during insonation. The tumor temperature was checked by inserting a thermistor probe (Anritsu Electric) into the central region of the tumor. It was kept below 35°C, much lower than the hyperthermia level.

Calibration of acoustic output from the transducer Although standing wave exposure modes were chosen for the relatively easy generation of reproducible cavitation in both *in vitro* and *in vivo* experiments, the output acoustic power from the transducer was calibrated in a free field (progressive wave mode) to avoid difficulty in acoustic power estimation. The output acoustic pressure was measured in degassed water 30 mm from the transducer surface using a 1-mm-diameter polyvinylidene difluoride needle-type hydrophone (Medicoteknisk Institut, Denmark). Spatial average intensity was calculated by scanning the probe, for 4 mm axially and laterally, to eliminate the effect of ripples in the field. The measured intensity
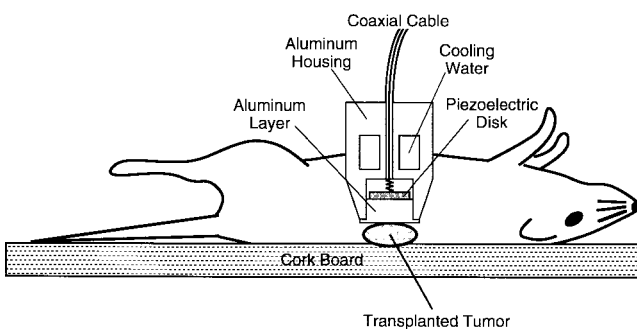

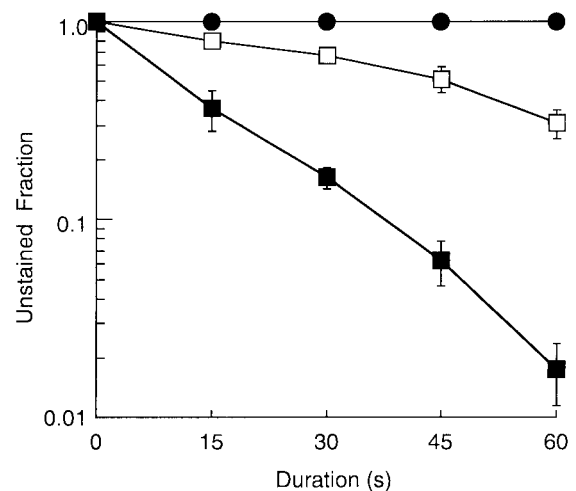Fig. 3. *In vivo* insonation set-up. A cross section of the transducer is shown.

Fig. 4. *In vitro* effect of ATX-S10 and/or ultrasound on isolated sarcoma 180 cells. ● ATX-S10 alone, □ ultrasound alone, ■ ATX-S10+ultrasound. Each point and vertical bar represent the mean±SD of four insonation experiments.
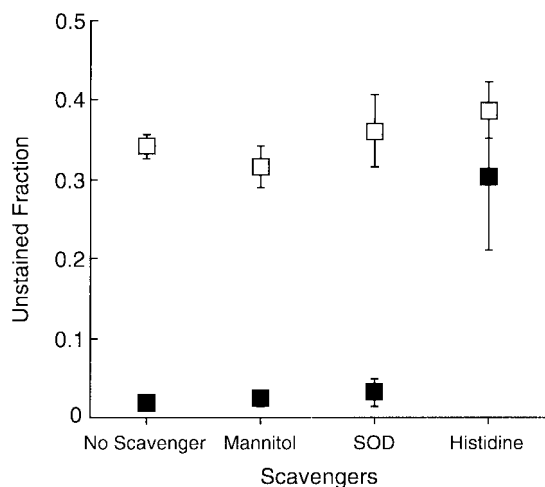
257

Fig. 5. Effect of active oxygen scavengers on cell damage with (■) and without (□) ATX-S10 after 60 s insonation. Each point and vertical bar represent the mean±SD of four insonation experiments.
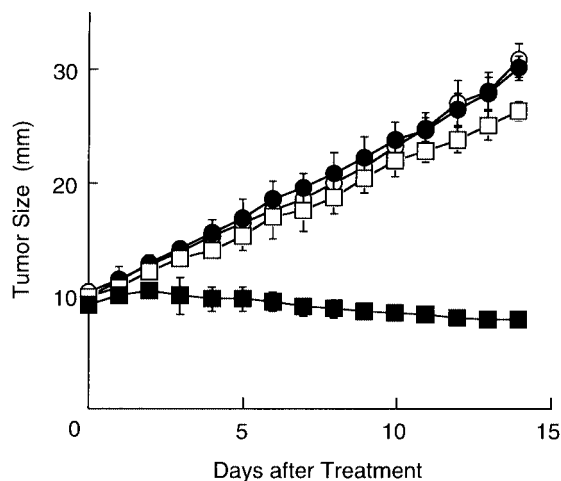

Fig. 6. Effect of ATX-S10 and/or ultrasound on the growth of colon 26 solid tumor. ○ control, ● ATX-S10 alone, □ ultrasound alone, ■ ATX-S10+ultrasound. Each point and vertical bar represent the mean±SD of four mice.

was approximately proportional to the square of the peak-to-peak driving signal voltage of the transducer in the voltage range used for the exposure. In both *in vitro* and *in vivo* experiments, the transducer was driven at a voltage corresponding to a certain free-field ultrasonic intensity, which is used to specify the exposure condition.

RESULTS

***In vitro* effect** The unstained fractions of the isolated sarcoma 180 cells in the air-saturated suspensions, in the presence and absence of 80 μM ATX-S10 after a fixed duration of insonation, are plotted versus insonation time in Fig. 4. The results with 80 μM ATX-S10 without ultrasound are also plotted versus time. The unstained fractions plotted on a logarithmic scale decreased linearly with insonation time. The ultrasonically induced cell-damaging rate was enhanced by ATX-S10 approximately two-fold. After 60 s insonation, the unstained fraction was reduced to 31% without ATX-S10, while it was only 1.8% in the presence of ATX-S10. No cell damage was observed with ATX-S10 alone.

The unstained fractions, after 60 s of insonation in the presence and absence of potential active oxygen scavengers, 10 mM histidine, 100 mg/ml SOD, or 100 mM mannitol, are compared in Fig. 5. Ultrasonically induced cell damage enhanced by 80 μM ATX-S10 was significantly reduced by histidine, but not significantly by either SOD or mannitol, while cell damage with ultrasound alone was not significantly reduced by either of these scavengers.

Table I. Antitumor Effect of ATX-S10 and/or Ultrasound on Colon 26

Treatment group	Tumor weight[a]	Inhibition ratio (%)[b]
Control	15.9±1.16	0
ATX-S10	15.4±1.22	3.31
Ultrasound	13.0±1.26	18.1
ATX-S10+ultrasound	3.86±0.59[c]	75.7

a) The values are the means+SD of four mice.
b) The inhibition ratio was calculated as (1−mean tumor weight of treatment group/mean tumor weight of control group)×100.
c) Significantly different ($P<0.05$) from control group.

Antitumor effect The effect of each treatment on the growth of colon 26 solid tumors is compared in Fig. 6 by plotting the tumor size for five weeks after the inoculation. ATX-S10 alone had no inhibitory effect on tumor growth. Ultrasound alone showed a slight inhibitory effect. ATX-S10+ultrasound showed a marked synergistic antitumor effect. Significant suppression of tumor growth after the treatment was observed in the combined treatment. The tumor weight after each treatment is shown in Table I. There is no significant difference among the control, ATX-S10 alone, and ultrasound alone. The tumor weight after the combination treatment was more than four times smaller than in the other three groups. The inhibition ratios were 3.31, 18.1 and 75.7% for the ATX-S10, ultrasound, and ATX-S10+ultrasound groups, respectively.

DISCUSSION

A significant ultrasonically induced antitumor effect as well as significant enhancement of ultrasonically induced *in vitro* cell damage was demonstrated with ATX-S10. ATX-S10 enhanced the ultrasonically induced damage to isolated sarcoma 180 cells by approximately the same factor as Hp at the same concentration.[7] In the experimental treatment combined with ultrasonic exposure, ATX-S10 inhibited the growth of the inoculated colon 26 tumors at a dose of 25 mg/kg. This dose level was lower than the LD_{50} by two orders of magnitude.[14] Colon 26 is a syngeneic experimental tumor and more suitable for evaluation of antitumor effects than sarcoma 180, which is allogeneic. This was the reason why colon 26 was used in the present *in vivo* experiment. On the other hand, sarcoma 180 was chosen for the *in vitro* experiment because it is difficult to obtain sufficiently high viability of colon 26 as isolated cells.

Histidine is known to act as a scavenger of singlet oxygen and possibly of hydroxyl radical. Thus, the significant reduction by histidine of ultrasonically induced cell damage enhanced by ATX-S10 suggests that the enhancement was due to ultrasonic generation of active oxygen enhanced by ATX-S10. The result may further suggest that not only the *in vitro* enhancement but also the ultrasonically induced *in vivo* antitumor effect with ATX-S10 was induced sonochemically. This should be confirmed in a further study.

Since a mannitol concentration of 100 mM is greater than the concentration reported to be effective to scavenge ultrasonically induced hydroxyl radical[17, 18] and no significant change in ultrasonically induced cell damage was observed with 100 mM mannitol, hydroxyl radical is not likely to be an important mediator of the damage. Superoxide radical may not be important either, since SOD had no significant effect. Among the active oxygen species, i.e., singlet oxygen, hydroxyl radical, and superoxide radical, singlet oxygen is therefore most likely to have mediated the ultrasonically induced cell damage enhanced with ATX-S10. Basically the same hypothesis (singlet oxygen as the mediator) has been proposed for Hp and ATX-70.[6, 17]

Sonochemically active cavitation inducing active oxygen generation is much less likely to take place inside the cells than outside. The resonant size of a microbubble in an aqueous medium at an ultrasonic frequency of the order of a megahertz is several micrometers. This is the same order of magnitude as the size of most mammalian tissue cells. Furthermore, the oxygen content in cytoplasm is lower by at least an order of magnitude than that in extracellular fluid, and the typical diffusion distance of an active oxygen species is less than 0.1 mm. Therefore, the cell membrane is most likely the site of action for sonochemical effects on cells subjected to ultrasound.

In conclusion, the presented results suggest that ATX-S10 is a potential sensitizer for sonodynamic tumor treatment. The results reported in this paper may be preliminary, but they support the possibility that clinical application of sonodynamic treatment using ATX-S10 will be feasible. Further investigations using experimental animals with a size similar to humans will be needed, however.

REFERENCES

1) Akimoto, R. An experimental study on the enhancement of the effect of anti-cancer drug by ultrasound. *J. Jpn. Soc. Cancer Ther.*, **3**, 562–570 (1985).
2) Kremkau, F. W. Cancer therapy with ultrasound. *J. Clin. Ultrasound*, **7**, 287–300 (1979).
3) Yumita, N., Okumura, A., Nishigaki, R., Umemura, K. and Umemura, S. The combination treatment of ultrasound and anti-tumor drugs on Yoshida sarcoma. *Jpn. J. Hyperthermia Oncol.*, **3**, 175–182 (1987).
4) Dougherty, T. J., Grindery, G. B., Weishaupt, K. R. and Boyle, D. G. Photoradiation therapy. II. Cure of animal tumors with hematoporphyrin and light. *J. Natl. Cancer Inst.*, **55**, 115–121 (1975).
5) Hayata, Y., Kato, H., Konaka, C., Ono, J. and Takizawa, N. Hematoporphyrin derivative and laser photoradiation in the treatment of lung cancer. *Chest*, **81**, 269–277 (1982).
6) Umemura, S., Yumita, N. and Nishigaki, R. Enhancement of ultrasonically induced cell damage by a gallium-porphyrin complex, ATX-70. *Jpn. J. Cancer Res.*, **84**, 582–588 (1993).
7) Yumita, N., Nishigaki, R., Umemura, K. and Umemura, S. Hematoporphyrin as a sensitizer of cell-damaging effect of ultrasound. *Jpn. J. Cancer Res.*, **80**, 219–222 (1989).
8) Kessel, D., Jeffers, R., Fowlkes, J. B. and Cain, C. A. Porphyrin-induced enhancement of ultrasound cytotoxicity. *Int. J. Radiat. Biol.*, **66**, 221–228 (1994).
9) Tachibana, K., Kimura, N., Okamura, M., Eguchi, H. and Tachibana, S. Enhancement of cell killing of HL-60 cells by ultrasound in the presence of the photosensitizing drug photofrin II. *Cancer Lett.*, **72**, 195–199 (1996).
10) Yumita, N., Nishigaki, R., Umemura, K. and Umemura, S. Synergistic effect of ultrasound and hematoporphyrin on sarcoma 180. *Jpn. J. Cancer Res.*, **81**, 304–308 (1990).
11) Umemura, S., Yumita, N., Nishigaki, R. and Umemura, K. Sonochemical activation of hematoporphyrin: a potential modality for cancer treatment. *Proc. 1989 IEEE Ultrason.*

Symp., 955–960 (1989).

12) Yumita, N., Sasaki, K., Umemura, S. and Nishigaki, R. Sonodynamically induced antitumor effect of a gallium-porphyrin complex, ATX-70. *Jpn. J. Cancer Res.*, **87**, 310–316 (1996).

13) Yumita, N., Nishigaki, R., Umemura, K., Morse, P. D., Swartz, H. M., Cain, C. A. and Umemura, S. Sonochemical activation of hematoporphyrin; an ESR study. *Radiat. Res.*, **138**, 171–176 (1994).

14) Nakajima, S., Hayashi, H., Sakata, I. and Takemura, K. New photosensitizer ATX-S10 having Type I photoreaction. *J. Clin. Exp. Med.*, **164**, 187–188 (1993) (in Japanese).

15) Nakajima, S., Hayashi, H., Omote, Y., Yamazaki, Y., Hirata, S., Maeda, T., Kobo, Y., Takemura, T., Kakiuchi, Y., Shindo, Y., Koshimizu, K. and Sakata, I. The tumor localizing properties of porphyrin derivatives. *J. Photochem. Photobiol. B: Biol.*, **7**, 189–198 (1990).

16) Nakajima, S., Maeda, T., Omote, Y., Hayashi, H., Yamazaki, K., Kobo, Y., Takemura, S., Shindo, Y. and Sakata, I. Tumor localizing Ga-porphyrin complex (ATX-70) as a new photosensitizer excited with YAG-laser. *J. Jpn. Soc. Laser Med.*, **10**, 225–228 (1989).

17) Umemura, S., Yumita, N., Nishigaki, R. and Umemura, K. Mechanism of cell damage by ultrasound in combination with hematoporphyrin. *Jpn. J. Cancer Res.*, **81**, 962–966 (1990).

18) Goldstein, S. and Czapski, G. Mannitol as OH scavenger in aqueous solutions and biological systems. *Int. J. Radiat. Biol.*, **46**, 725–729 (1984).