Acridine Orange Used for Photodynamic Therapy Accumulates in Malignant Musculoskeletal Tumors Depending on pH Gradient

TAKAO MATSUBARA, KATSUYUKI KUSUZAKI, AKIHIKO MATSUMINE, KEN SHINTANI, HARUHIKO SATONAKA and ATSUMASA UCHIDA

Department of Orthopedic Surgery, Mie University Faculty of Medicine, Mie, Japan

Abstract. *Background: Intra-operative photodynamic therapy has been applied with acridine orange (AO-PDT) to human musculoskeletal sarcomas for the past 4 years, resulting in a low local recurrence rate, within 10%, after intra-marginal tumor resection and excellent limb function. However, it is still unclear why acridine orange (AO) specifically accumulates in tumor cells, especially in malignant tumor cells. The purpose of this study was to clarify the mechanism of AO accumulation in malignant musculoskeletal tumors. Materials and Methods: Sixty-two musculoskeletal tumors, including 35 malignant and 27 benign tumors, were studied. Using freshly resected tumor material, the extracellular pH (pHe) was measured and the fluorescence intensity of AO accumulated in the tumors was measured by an image analyzer after ex vivo exposure to 1.0 μg/ml AO, followed by blue excitation. In the in vitro study, bafilomycin A1 was exposed to LM 8 mouse osteosarcoma cells, in order to inhibit V-ATPase, subsequently causing a decrease in the pH gradient (ΔpH) between the intracellular pH (pHi) and extracellular pH (pHe) or between the pHi and the pHe. AO accumulation and the cytocidal effect of AO were evaluated. Results: The results of the in vivo study, using human materials freshly resected at surgery, revealed that the pHe of the malignant musculoskeletal tumors was significantly lower than that of the benign tumors and normal muscles or adipose tissues and also showed that the AO fluorescence intensity of the malignant musculoskeletal tumors was significantly stronger than that of the benign tumors and normal muscles or adipose tissues. The results also revealed that the AO fluorescence intensity negatively correlated with the pHe in tumors and normal tissues. The in vitro study showed that bafilomycin A1 inhibited the accumulation of AO in acidic organelles, such as lysosomes, and that the cytocidal effect of AO-PDT was also remarkably inhibited. Discussion: Based on results of the in vivo and in vitro studies, it is suggested that malignant musculoskeletal tumors have a large ΔpH between the pHi and the pHe or between the pHi and the vaculolar pH and also that a large ΔpH increases AO accumulation in tumors. We, therefore, believe that AO-PDT may be more effective in highly malignant musculoskeletal tumors than low grade ones, because of their acidity. Conclusion: AO accumulation in musculoskeletal tumors was dependent on the ΔpH between the pHi and the pHe, or between the pHi and the vacuolar pH.*

Acridine orange (AO) has been found to specifically accumulate in musculoskeletal sarcomas and to emit green fluorescence with a strong cytocidal effect on mouse osteosarcoma cells after illumination with blue excitation light. Based on *in vitro* and *in vivo* basic research studies (1-11), a human therapy was designed. We developed a new photodynamic therapy in which AO acts as a photosensitizer (AO-PDT), in order to preserve excellent limb function after reduction surgery with intralesional or marginal tumor resection. Since 1999, we have been using AO-PDT to treat patients with musculoskeletal sarcomas, and have achieved a local recurrence rate below 10% and excellent limb function, including running, jumping and throwing (12-14). However, it remains unclear why AO specifically accumulates in tumor cells, especially malignant tumor cells. AO is a basic dye and densely accumulates in lysosomes (15-18), which are strongly acidic organelles, and it is generally assumed that extracellular

Abbreviations: ΔpH, pH gradient; AO, acridine orange; AO-PDT, photodynamic therapy with acridine orange; pHe, extracellular pH; pHi, intracellular pH; V-ATPase, vacuolar-ATPase; LM 8, murine osteosarcoma cell line; MPNST, malignant peripheral nerve sheath tumor; MFH, malignant fibrous histiocytoma; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

Correspondence to: Katsuyuki Kusuzaki, MD, Department of Orthopaedic Surgery, Mie University Faculty of Medicine, Edobashi 2-174, Tsu city, Mie Prefecture 514-8507, Japan. Tel: +81 59 231 5022, Fax: +81 59 231 5211, e-mail: kusu@clin.medic.mie-u.ac.jp

Key Words: Musculoskeletal sarcoma, photodynamic therapy, acridine orange, pH, fluorescence.

pH (pHe) is more acidic in tumors than in normal tissues, because tumors produce acidity, in the form of protons (H^+), in a hypoxic environment (19, 20). Based on these findings, we hypothesize that AO accumulates in tumor cells, due to the acidic conditions around the tumor. In the present study, the relationship between AO accumulation and the pHe of human musculoskeletal tumors and normal tissues was investigated and an *in vitro* experiment was performed to clarify the relationship between AO accumulation and the pH gradient (ΔpH) between intracellular and extracellular fluid.

Materials and Methods

In vivo study

a) Materials: Sixty-two musculoskeletal tumors, including 27 benign tumors and 35 malignant tumors, were used. The 27 benign tumors comprised 11 lipomas, 5 schwannomas, 3 desmoid tumors, 3 neurofibromas, 2 giant cell tumors of bone, 1 hemangioma, 1 angioleiomyoma and 1 enchondroma. The 35 malignant tumors comprised 9 malignant fibrous histiocytomas (MFH), 6 chondrosarcomas, 3 leiomyosarcomas, 2 malignant peripheral nerve sheath tumors (MPNST), 2 rhabdomyosarcomas, 2 synovial sarcomas, 1 osteosarcoma, 1 unclassified spindle cell sarcoma, 1 chordoma, 1 Ewing's sarcoma, 1 clear cell sarcoma, 1 malignant granular cell tumor and 1 dermatofibrosarcoma protuberance. Eleven normal skeletal muscles and 14 normal adipose tissue samples, obtained from inter-muscle or subcutis regions of widely resected tumors, were also used.

b) Measurement of pHe in the tumor tissues: Fresh tumor samples were obtained during surgical tumor resection and were immediately used under sterile conditions. Measurement of the pHe required a tumor with a diameter greater than 3 cm.

For measurement of the pHe in tumors and normal tissue, an MP120 Basic Portable pH Meter (Mettler Toledo, S.A., France) was used. The pHe was measured immediately after tumor resection, according to the following procedure. After cutting of the resected tumor at its maximum diameter, the pH meter was inserted deeper than 1 cm from the cut surface. The pHe was measured in 3 different areas, avoiding necrotic or hemorrhagic lesions.

c) Measurement of AO fluorescence intensity in the tumor tissues. The fresh tumor material used to measure the pHe was exposed to a 1 μg/ml AO (Sigma-Aldrich Chemie Gmbh, Germany: Lot No.122K0522) solution dissolved in phosphate-buffered saline (PBS; pH 7.4) for 3 minutes, and was then washed 3 times with PBS. The cut surface of the tumor material was then illuminated with 10,000-lux blue light (450-490 nm) from a 150-Watt xenon lamp (SAN-EI Electronics Co., Ltd., Tokyo, Japan). The contrast in fluorescence between the tumor and surrounding muscle was observed using a fluorescence stereoscope SMZ1000 (Nikon Co., Ltd., Kanagawa, Japan) with an absorption filter (>520 nm). Fluorescence images were made using a digital camera C-5050 (Olympus Co., Ltd., Tokyo, Japan) and the image data was input into a personal computer as gray-scale. The fluorescence intensity ratio (X) was calculated between the tumor (A) and the background (B) using Scion Image Beta analysis software (Figure 1).

In vitro study

a) Cell line: A murine osteosarcoma cell line, that readily metastasizes to the lung (LM8) (21), was established from Dunn's osteosarcoma cell line. The LM8 cell line is expected to have strongly acidic organelles. LM8 was harvested in DMEM containing 10% fetal bovine serum at 37˚C under a 5% CO_2 atmosphere.

b) Influence of bafilomycin A1 on the uptake of AO: Bafilomycin A1 down-regulates the ΔpH between the intra- and extra-cellular fluid by inhibiting V-ATPase (22-25). LM8 cells ($1x10^5$), cultured in 5-cm plates, were exposed to 10 nM bafilomycin A1 in DMEM conditioned at pH 7.4 for 24 h, and were then exposed to 1 μg/ml AO for 10 min. The cells were subsequently washed with PBS (pH 7.4) 3 times, and 5 ml of 2-propanol with 10 mM HCl were then added to dissolve the AO in the cells. The fluorescence intensity of these extract solutions (100 μl) was measured using a micro-plate spectrofluorometer Multiscan JX (Thermo Labsystems, Kanagawa, Japan) at 492 nm excitation wavelength.

c) Effect of bafilomycin on the cytocidal activity of AO-PDT. LM8 cells ($1x10^4$) cultured in 16 96-well plates with DMEM (pH 7.4) were exposed to 10 nM bafilomycin A1 for 24 h, followed by exposure to 0.1 μg/ml AO for 10 minutes. Cells containing AO were illuminated with unfiltered light from a 500-Watt xenon lamp (SAN-EI Electronics Co.), but the control group cells were not illuminated. After illumination for 10 min, the tumor cells were continuously cultured in medium containing AO and bafilomycin A1. The viable cells in each well were counted at 30 min, 24, 48 and 72 h after illumination. Phototoxicity was assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] assay, which measures mitochondrial dehydrogenase activity, according to the manufacturer's instructions (Promega Corporation, USA).

Results

In vivo study

a) pHe in the tumor tissues: The average pHe was 7.16 (±0.25) in the 27 benign musculoskeletal tumors, 6.78 (±0.26) in the 35 malignant musculoskeletal tumors, 7.26 (±0.14) in the normal muscles and 7.43 (±0.11) in the normal adipose tissues (Figure 2). The malignant tumors were significantly more acidic than the benign tumors ($p<0.05$) and normal tissues ($p<0.01$).

b) AO fluorescent intensity in the tumor tissues: The average fluorescence intensity ratio (X) was 3.64 (±1.67) in the 27 benign tumors, 5.56 (±2.56) in the 35 malignant tumors, 1.57 (±0.48) in the normal muscles and 1.43 (±0.78) in the adipose tissues (Figure 3). The AO fluorescence intensity, which correlates with the AO accumulation in the tumor, was significantly higher in the malignant tumors than in the benign tumors ($p<0.05$) or the normal tissues ($p<0.01$).

c) Relationship between pHe and AO fluorescence intensity in the tumor tissues: The relationship between the fluorescence intensity ratio of AO (X) and the pHe of tumors and normal tissues is shown in Figure 4. The AO fluorescence intensity ratio negatively correlated with the pHe in the tumors and normal tissues. Pearson's correlation coefficient

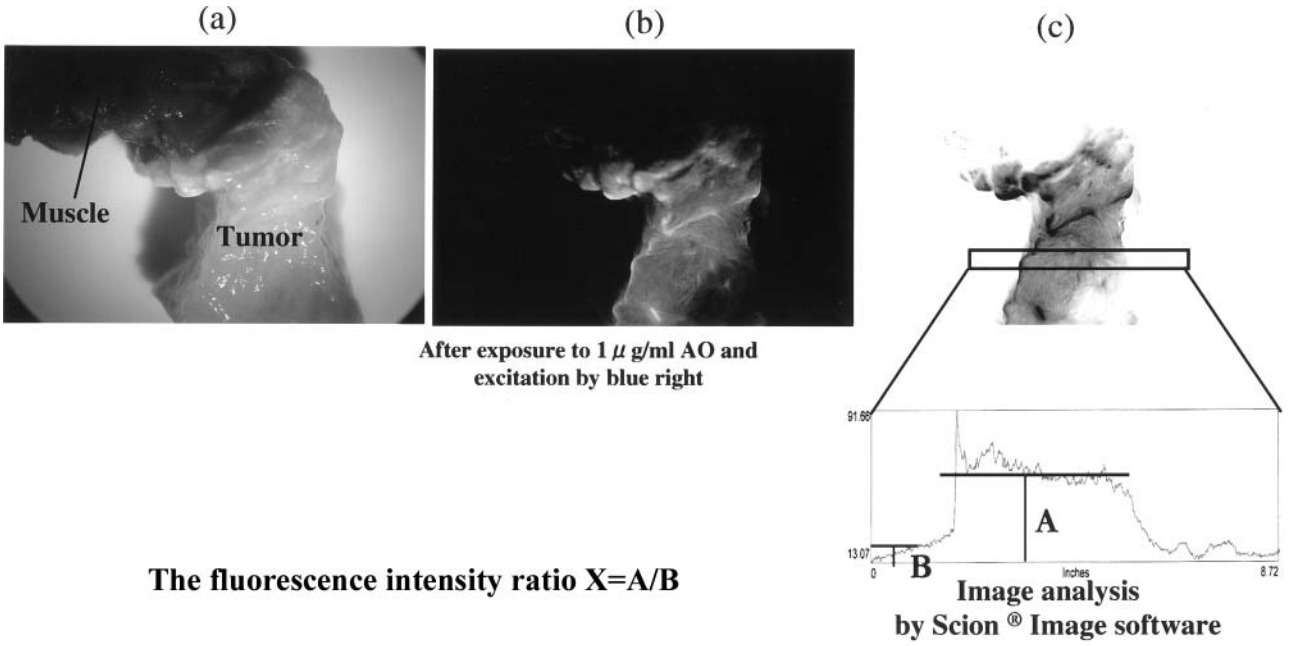

Figure 1. *Method of measurement of AO fluorescence intensity. These fresh tumor materials (a) incubated with 1 μg/ml AO were illuminated by a halogen lamp (b). The contrast in fluorescence (observed through an absorption filter) between the tumor and surrounding muscle was input into a personal computer as a gray scale (c) and was quantified as fluorescence brightness by imaging software. The fluorescence intensity ratio (X) between tumor (A) and background (B) was calculated.*

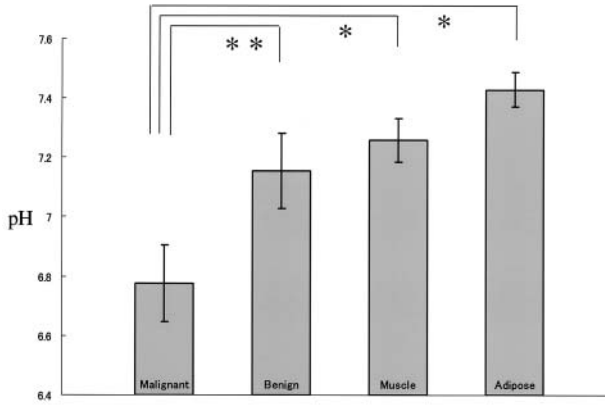

Figure 2. *The pHe of malignant and benign tumors, muscle and adipose tissues. The malignant tumors were significantly more acidic than the benign tumors (**p<0.05) and the normal tissues (*p<0.01).*

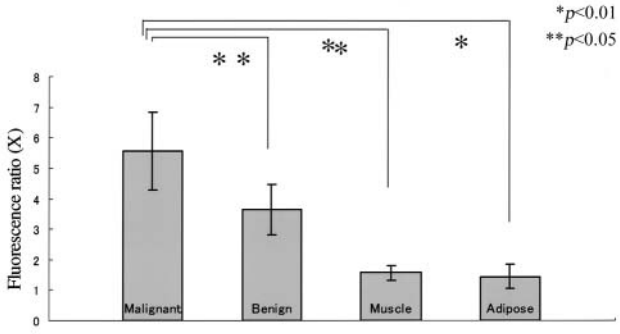

Figure 3. *The fluorescence intensity ratio (X) of malignant and benign tumors, muscle and adipose tissues. The AO fluorescence intensity, which correlates with AO accumulation in the tumor, was significantly higher in the malignant tumors than in the benign tumors (**p<0.05) or the normal tissues (*p<0.01).*

was –0.621 ($p<0.01$). Intense accumulation of AO was observed in MPNST, rhabdomyosarcoma, chondrosarcoma, leiomyosarcoma, giant cell tumor of bone and desmoid tumor (Figure 5).

In vitro study

Effect of bafilomycin A1 on AO uptake and cytocidal activity of AO-PDT: The uptake of AO by LM8 cells exposed to 0.1 μg/ml AO decreased by one-third after pre-exposure to bafilomycin

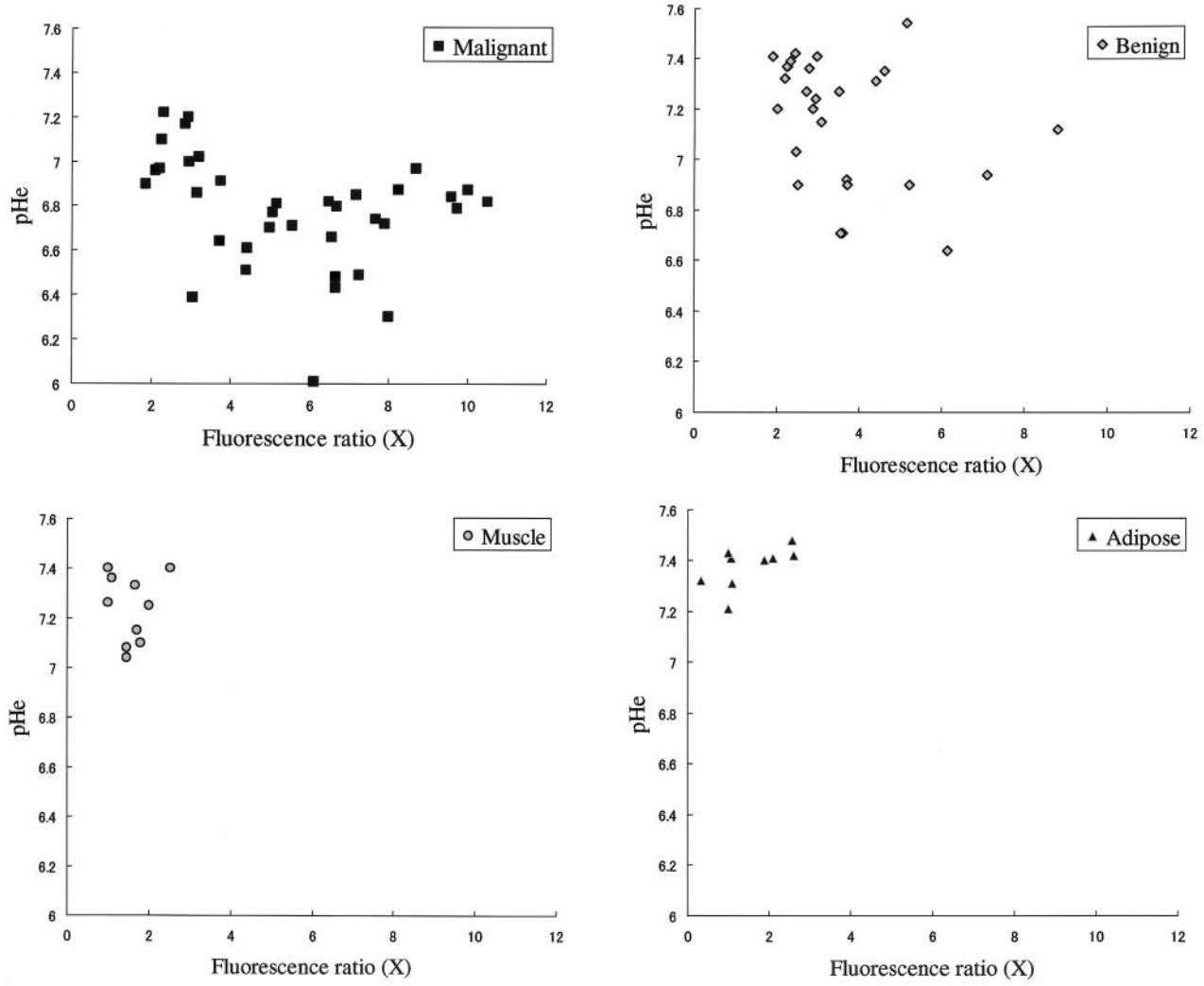

Figure 4. *Relationship between the AO fluorescence ratio and the pHe of malignant and benign tumors, muscle and adipose tissues. The AO fluorescence intensity ratio negatively correlated with the pHe in the tumors and normal tissues. Pearson's correlation coefficient was –0.621 ($p<0.01$).*

A1 ($p<0.01$, Figure 6). The viability ratio of LM8 cells exposed to 0.1 μg/ml AO also markedly decreased after pre-exposure to bafilomycin A1 ($p<0.01$, Figure 7).

Discussion

We recently developed a novel AO-PDT and have used it to treat patients with musculoskeletal sarcomas during reduction surgery with marginal or intralesional tumor resection. We have obtained a recurrence rate below 10% for patients primarily treated with AO-PDT, and have obtained excellent limb function, including running, jumping and throwing (12-14). Before clinical application of AO-PDT, our experimental studies revealed that AO has the following unique biological activities: a) AO rapidly accumulates in viable mouse osteosarcoma cells and emits green fluorescence from the cytoplasm and orange fluorescence from the lysosome after blue-light illumination; b) AO specifically accumulates in primary and metastatic lesions of mouse osteosarcoma, enabling visualization of the tumor by fluorescence microscopy; c) AO has a strong cytocidal effect after excitation by blue or unfiltered light from a xenon lamp (7-11). The cytocidal effect of AO-PDT is thought to be due to the singlet oxygen produced from triplet oxygen *via* light excitation of AO (photochemical reaction type II) (6, 7, 10, 26). Singlet oxygen is toxic to all intracellular structures. The results of our morphological study suggest that AO binds to lysosomes (emitting orange fluorescence) and all types of RNA (emitting green fluorescence) in living mouse osteosarcoma cells (11), but details of this AO binding are unknown. We found that, although AO quickly accumulates in mouse osteosarcoma cells as well as in normal cells in muscles or adipose tissue after

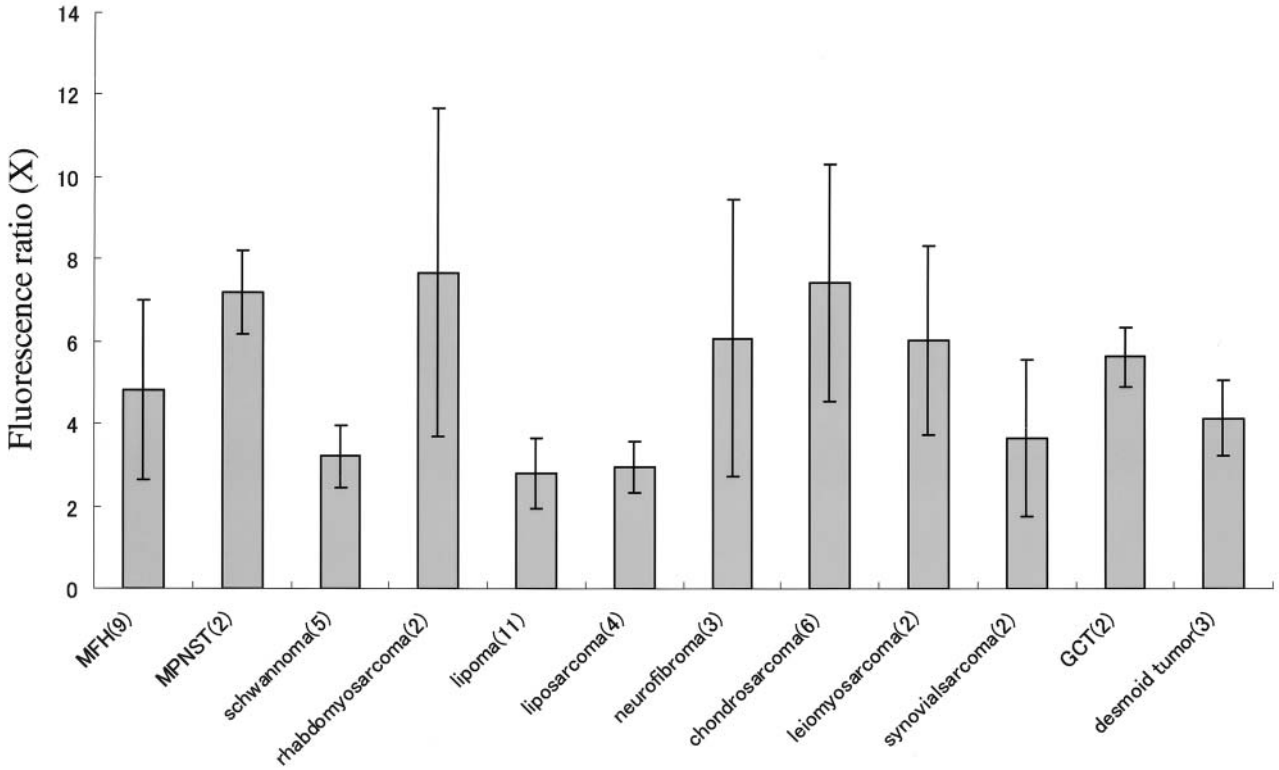

Figure 5. *The average AO fluorescence intensity ratio in malignant and benign musculoskeletal tumors. Intense accumulation of AO was observed in MPNST, rhabdomyosarcoma, chondrosarcoma, leiomyosarcoma, giant cell tumor of bone and desmoid tumor. The number inside parentheses shows the case number of tumors. MFH: malignant fibrous histiocytomas; MPNST: malignant peripheral nerve sheath tumor; GCT: giant cell tumor.*

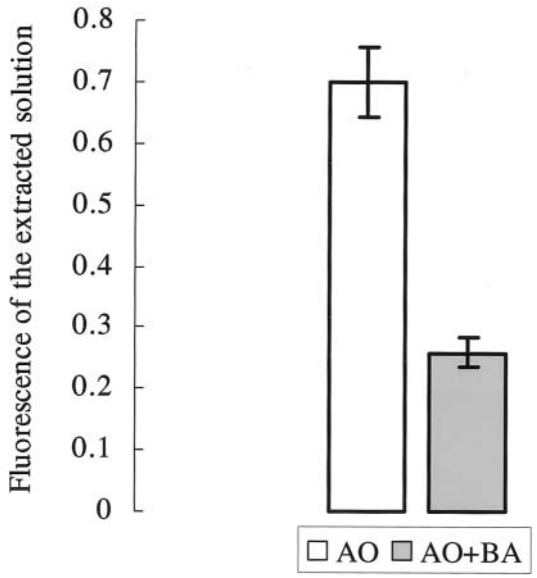

Figure 6. *AO uptake in murine osteosarcoma cells (LM8) with or without bafilomycin A1. AO: acridine orange without bafilomycin A1; AO+BA: acridine orange with bafilomycin A1; numerical value of the Y-axis show fluorescence of the extracted solution (100 μl) in direct proportion to the level of AO in the solution (n=6). This figure shows that uptake of AO by LM8 cells exposed to 0.1 μg/ml AO decreased by one-third after pre-exposure to bafilomycin A1 (p<0.01).*

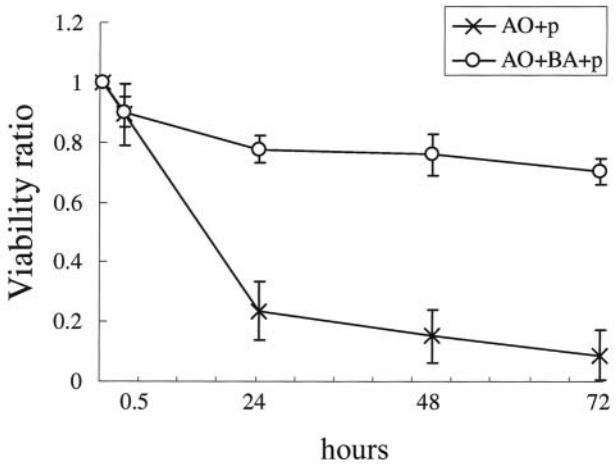

Figure 7. *Cytocidal effect of AO-PDT on murine osteosarcoma cells (LM8) with or without bafilomycin A1. AO+p: group of AO-PDT without pre-exposure to bafilomycin A1; AO+BA+p: group of AO-PDT with pre-exposure to bafilomycin A1. In AO-PDT to cultured cells, the viability ratio of LM8 cells exposed to 0.1 μg/ml AO also markedly decreased after pre-exposure to bafilomycin A1 (p<0.01).*

intraperitoneal injection, AO was subsequently excluded from normal cells within 2 h, but not from tumor cells. Therefore, AO remains in the tumor cells longer than in the normal cells (7-10). However, details of the mechanism by which AO accumulates in malignant tumor cells are unknown. There are many candidate photosensitizers for PDT in the surgical treatment of cancer, but most do not specifically accumulate in tumors. Even derivatives or precursors of hematoporphyrin, which are widely used in PDT for cancer surgery, accumulate in both tumor cells and normal cells *e.g.* endothelial cells or squamous cells) (27). Avoiding damage to normal tissues is an important issue in PDT and clarification of the mechanism of the tumor-specific accumulation of AO may lead to the development of a novel photosensitizer with superior effectiveness and safety.

AO is a weakly basic dye commonly used for fluorescence staining of DNA (green fluorescence) and RNA (red fluorescence) in fixed cells; green and red fluorescence are measured in individual cells by flow cytometry or fluorocytometry (28). As described above, in viable cells, AO sparsely accumulates in the cytoplasm (emitting green fluorescence) and densely accumulates in lysosomes (emitting orange fluorescence) (11). There is no evidence that AO does bind to the mitochondria or DNA in living cells, but AO binds to nucleoli, suggesting that AO binds all RNA, mainly transfer RNA in the cytoplasm (26). Lysosomes are strongly acidic organelles. AO also accumulates in acidic vacuoles of living organisms such as bacteria and malaria parasites (29, 30). These findings suggest that AO accumulation is closely related to acidity. Therefore, in the present study, we examined the relationship between pH and fluorescence intensity in musculoskeletal tumors. The measurement of pHi using resected tumor materials is not easy, due to the microinjection technique, but pHe can be measured using the pen-type detector employed in the present study. The results of the pilot study revealed that the pHe of most musculoskeletal sarcomas, which emitted strong green fluorescence after *ex vivo* exposure to AO followed by blue-light excitation, tended to be lower than that of the surrounding muscular or adipose tissues, which emitted no fluorescence or weak green fluorescence.

The results of the present *in vivo* study revealed that the extracellular fluid of the malignant musculoskeletal tumors was significantly more acidic than that of the benign tumors, normal muscle tissue and adipose tissue, and also showed that the AO fluorescence intensity of the malignant musculoskeletal tumors was significantly stronger than that of the benign tumors, normal muscle tissue and adipose tissue. Furthermore, AO fluorescence intensity was found to negatively correlate with the pHe in tumors and normal tissues. It is widely believed that malignant tumors are more acidic than benign tumors, but there is controversy regarding the mechanism responsible for this difference. Malignant tumor cells actively produce protons (H^+) because of increasing levels of lactate or CO_2 in a hypoxic environment. However, many intracellular activities require maintenance of the pHi within a narrow range (7.2 ~ 7.4). Consequently, malignant tumor cells pump H^+ into the extracellular fluid *via* vacuolar-ATPases, an Na^+-H^+ exchanger, or a monocarboxylate carrier (20). In malignant musculoskeletal tumors, because of the low pHe and a narrow range of pHi, there is a large ΔpH between the pHi and the pHe and between the pHi and the intravacuolar pH (20, 31). We, therefore, hypothesized that AO accumulates in environments with a large ΔpH. This hypothesis is supported by the results of the present *in vitro* study using bafilomycin A1, which inhibited V-ATPase and decreased the ΔpH of intracellular acidic organelles (22-25, 32, 33). These results show that bafilomycin A1 inhibited the accumulation of AO in acidic organelles, such as lysosomes, and that bafilomycin A1 also markedly inhibited the cytocidal effect of AO-PDT. In another study, it was shown that NH_4Cl, which reduces the acidity of intracellular vacuoles, reduced the fluorescence emission by intracellular AO (34). These findings suggest that an extremely low ΔpH causes a decrease in AO accumulation and that a large ΔpH increases AO accumulation.

A previous study indicated that invasion by cancer cells is facilitated by their secretion of catabolic enzymes that are sequestered in lysosomal vesicles, and that breast cancer cells of different degrees of malignancy are frequently characterized by extracellular acidity (35). The low pHe observed in that study reflected the increasing number of lysosomes, and larger lysosomal vesicles were observed more frequently in highly metastatic breast cancer cells. In another study, multidrug-resistant cancer cells incubated with AO were found to have high AO fluorescence intensity (34). These findings suggest that highly malignant tumors exhibiting metastases, local invasion or chemoresistance are more acidic and contain many acidic vacuoles. If this is true, it indicates that AO accumulates in highly malignant tumors at a greater rate than in tumors of low-grade malignancy, and indicates that the cytocidal effect of AO is increased by PDT.

Based on results of the present *in vivo* and *in vitro* experiments, we conclude that malignant musculoskeletal tumors have a large ΔpH between the pHi and the pHe and between the pHi and the vacuolar pH, and that a large ΔpH increases AO accumulation in tumors. We also conclude that AO-PDT is more effective against highly malignant musculoskeletal tumors, because of the greater acidity in these tumors.

References

1 Korgaonkar K and Sukhatankara J: Anti-tumor activity of the fluorescent dye, acridine orange, on Yoshida sarcoma (ascites). Br J Cancer *17*: 471-473, 1963.

2 Kato A: Gastrofiberscopic diagnosis with acridine orange fluorescence. Gastroenterol Endosc *12*: 351-362, 1970.

3 Tomson SH, Emmett EA and Fox SH: Photodestruction of mouse epithelial tumors after oral acridine orange and argon laser. Cancer Res *34*: 3124-3127, 1974.
4 Tatsuta M, Yamamura H, Yamamoto R, Ichii M, Noguchi S, Iishi H, Mishima H, Hattori T and Okuda S: Destruction of implanted gastric tumors in rats by acridine orange photoactivation with an argon laser. Eur J Cancer Clin Oncol *20*: 543-552, 1984.
5 Ishikawa S, Nemoto R, Kanoh S, Kobayashi K and Ishizaka S: Photodynamic inactivation of bladder cancer cells (MGH-U1) sensitized with acridine orange and irradiated by argon laser. Tohoku J Exp Med *144*: 265-2671, 1984.
6 Zdolsek JM: Acridine orange-mediated photodamage to cultured cells. Apmis *101*: 127-132, 1993.
7 Kusuzaki K, Minami G, Takeshita H, Murata H, Hashiguchi S, Nozaki T, Ashihara T and Hirasawa Y: Photodynamic inactivation with acridine orange on a multidrug-resistant mouse osteosarcoma cell line. Jpn J Cancer Res *91*: 439-445, 2000.
8 Kusuzaki K, Aomori K, Suginoshita T, Minami G, Takeshita H, Murata H, Hashiguchi S, Ashihara T and Hirasawa Y: Total tumor cell elimination with minimum damage to normal tissues in musculoskeletal sarcomas by photodynamic reaction with acridine orange. Oncology-Bazel *59*: 174-180, 2000.
9 Kusuzaki K, Suginoshita T, Minami G, Aomori K, Takeshita H, Murata H, Hashiguchi S, Ashihara T and Hirasawa Y: Fluorovisualization effect of acridine orange on mouse osteosarcoma. Anticancer Res *20*: 3019-3024, 2000.
10 Hashiguchi S, Kusuzaki K, Murata H, Takeshita H, Hashiba M, Nishimura T, Ashihara T and Hirasawa Y: Acridine orange excited by low-dose radiation has a strong cytocidal effect on mouse osteosarcoma. Oncology-Bazel *62*: 85-93, 2000.
11 Kusuzaki K, Murata H, Takeshita H, Hashiguchi S, Nozaki T, Emoto K, Ashihara T and Hirasawa Y: Intracellular binding sites of acridine orange in living osteosarcoma cells. Anticancer Res *20*: 971-976, 2000.
12 Yoshida K, Kusuzaki K, Matsubara T, Matsumine A, Kumamoto T, Komada Y, Naka N and Uchida A: Periosteal Ewing's sarcoma treated by photodynamic therapy with acridine orange. Oncol Rep *13*: 279-282, 2005.
13 Kusuzaki K, Murata H, Matsubara T, Miyazaki S, Okamura A, Seto M, Matsumine A, Hosoi H, Sugimoto T and Uchida A: Clinical outcome of a new photodynamic therapy with acridine orange for synovial sarcomas. Photochem Photobiol *81*: 705-709, 2005.
14 Kusuzaki K, Murata H, Matsubara T, Miyazaki S, Okamura A, Seto M, Matsumine A, Hosoi H, Sugimoto T and Uchida A: Clinical trial of photodynamic therapy using acridine orange with/without low dose radiation as new limb salvage modality in musculoskeletal sarcomas. Anticancer Res *25*: 1225-1236, 2005.
15 Zelenin AV: Fluorescence microscopy of lysosomes and related structures in living cells. Nature *212*: 425-426, 1966.
16 Schmidt H and Rosenkranz P: On the mechanism of the acridine orange sensitized photodynamic inactivation of lysozyme I. Basic kinetics. Z Naturforsch [C] *31*: 29-39,1976.
17 Rosenkranz P and Schmidt H: On the mechanism of the acridine orange sensitized photodynamic inactivation of lysozyme. II. Kinetics in presence of N-acetylglucosamine. Z Naturforsch [C] *31*: 679-682, 1976.
18 Zdolsek JM, Olsson GM and Brunk UT: Photooxidative damage to lysosomes of cultured macrophages by acridine orange. Photochem Photobiol *51*: 67-76, 1990.
19 Webb SD, Sherratt JA and Fish RG: Mathematical modeling of tumour acidity: regulation of intracellular pH. J Theor Biol *196*: 237-250, 1999.
20 Stubbs M, McSheehy PM, Griffiths JR and Bashford CL: Causes and consequences of tumour acidity and implications for treatment. Mol Med Today *6*: 15-19, 2000.
21 Asai T, Ueda T, Ito K, Yoshioka K, Aoki Y, Mori S and Yoshikawa H: Establishment and characterization of a murine osteosarcoma cell line (LM8) with high metastatic potential to the lung. Int J Cancer *76*: 418-422, 1998.
22 Bowman EJ, Siebers A and Altendorf K: Bafilomycins: a class of inhibitors of membrane ATPases from microorganisims, animal cells, and plant cells. Proc Natl Acad Sci USA *85*: 7972-7976, 1988.
23 Yoshimori T, Yamamoto A, Moriyama Y, Futai M and Tashiro Y: Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J Biol Chem *266*: 17707-17712, 1991.
24 Tapper H and Sundler R: Bafilomycin A1 inhibits lysosomal, phagosomal, and plasma membrane H(+)-ATPase and induces lysosomal enzyme secretion in macrophages. J Cell Physiol 163: 137-144, 1995.
25 Sennoune SR, Luo D and Martinez-Zaguilan R: Plasmalemmal vacuolar-type H+-ATPase in cancer biology. Cell Biochem Biophys *40*: 185-206, 2004.
26 Amagasa J: Mechanisms of photodynamic inactivation of acridine orange-sensitized transfer RNA: participation of singlet oxygen and base damage leading to inactivation. J Radiat Res (Tokyo) *27*: 339-335, 1986.
27 Lim HW: Effects of porphyrins on skin. Ciba Found Symp *146*: 148-153; discussion 154-158, 1989.
28 Kapuscinski J, Darzynkiewicz Z and Melamed MR: Interactions of acridine orange with nucleic acids. Properties of complexes of acridine orange with single stranded ribonucleic acid. Biochem Pharmacol *32*: 3679-3694, 1983.
29 Zampieri A and Greenberg J: Mutagenesis by acridine orange and proflavin in *Escherichia coli* strain S. Mutat Res *2*: 552-556, 1965.
30 Rickman L, Long G, Oberst R, Cabanban A, Sangalang R, Smith J, Chulay J and Hoffman S: Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. Lancet *14*: 68-71, 1989.
31 Dell'Antone P, Bragadin M and Piergallini L: Lonidamine cytotoxicity: involvement of the lysosomal component. J Int Med Res *28*: 269-276, 2000.
32 Kurashima K, Numata M, Yachie Y, Sai Y, Ishizaka N, Fujimura M, Matsuda T and Ohkuma S: The role of vacuolar H+-ATPase in the control of intragranular pH and exocytosis in eosinophils. Lab Invest *75*: 689-698, 1996.
33 Wada S, Kantha S, Yamashita T, Matsunaga S, Fusetani N and Watabe S: Accumulation of H+ in vacuoles induced by a marine peptide toxin, theonellamide F, in rat embryonic 3Y1 fibroblasts. Mar Biotechnol (NY) *4*: 571-582, 2002.
34 Millot C, Millot JM, Morjani H, Desplaces A and Manfait M: Characterization of acidic vesicles in multidrug-resistant and sensitive cancer cells by acridine orange staining and confocal microspectrofluorometry. J Histochem Cytochem *45*: 1255-1264, 1997.
35 Glunde K, Guggino SE, Solaiyappan M, Pathak AP, Ichikawa Y and Bhujwalla ZM: Extracellular acidification alters lysosomal trafficking in human breast cancer cells. Neoplasia *6*: 533-545, 2003.

Received August 12, 2005
Accepted October 6, 2005