

# Intraoperative Photodynamic Surgery (iPDS) with Acridine Orange for Musculoskeletal Sarcomas

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## Abstract

We recently established the new limb salvage modality of acridine orange (AO) therapy (AOT), in an attempt to develop minimally invasive limb salvage surgery with minimal damage of normal tissues and a low risk of local recurrence. The treatment modality consists of intraoperative photodynamic surgery (iPDS) and photodynamic therapy (iPDT), followed by postoperative radiodynamic therapy (RDT) using AO for patients with high-grade malignant musculoskeletal sarcomas. Clinical results have shown that the treatment is associated with a low risk of local recurrence, the risk being almost the same as that following conventional wide resection, and yields superior limb function as compared to that obtained after wide resection.

In this review, we present the detailed mechanism of selective accumulation of AO in sarcomas, which is related to the acidic environment and lysosomal acidity of the tumor cells induced by cancer-specific glycolysis not involving the define tricarboxylic acid (TCA) cycle (Warburg's effect). We also describe the clinical uses of AOT and the procedure for intraoperative photodynamic surgery (iPDS) using local administration of AO.

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**Categories:** General Surgery, Oncology, Orthopedics

**Keywords:** acridine orange, photodynamic therapy, photodynamic surgery, musculoskeletal sarcomas, cancer acidity, lysosomes, photodynamic diagnosis, radiodynamic therapy

## Introduction And Background

For all tumor surgeons in every medical area, it would be a dream to accurately visualize the tumor during surgical resection. It would enable easy and complete removal of the tumor mass without excessive damage of normal tissues. It would result in good functioning of organs or tissues. However, no such ideal method has been devised yet, although there are scattered reports of the use of various kinds of fluorescence dyes towards achieving this purpose [1-5]. The present review paper introduces one such ideal agent that could aid surgeons in realizing their dream: acridine orange (AO).

AO is not a newly synthesized product. It was first extracted from coal tar in the late 19th century, as a weak basic dye for dyeing clothes or staining microorganisms. AO has many unique biological activities, as previously reported, such as antitumor activity, photosensitizing activity, pH-detecting activity, fluorescence emission, staining activity in sperm, bacteria, viruses, parasites, and fungi [6-14]. AO also has the unique feature of metachromasia, emitting green fluorescence from the monomer form and orange fluorescence from the dimer form following blue light excitation [7].

Since AO has a simple chemical structure and a very low molecular weight (M.W. 265) (Figure 1), it has the ability to rapidly flow into the cytoplasm of living cells through the plasma membrane by passive diffusion and to bind to a variety of RNAs and lysosomes [15].

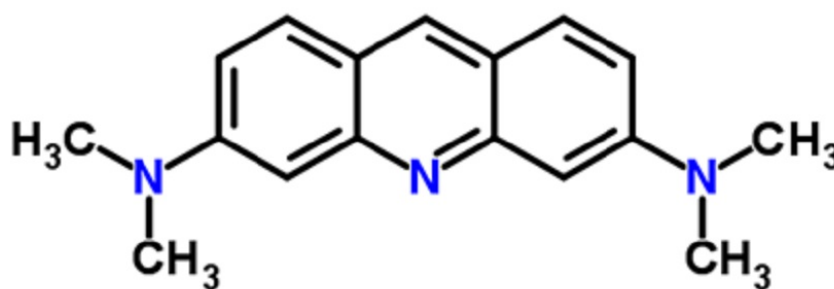
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Molecular weight: 265

**FIGURE 1: Chemical structure of acridine orange (AO)**

AO accumulates especially under acidic conditions, because it is a weak basic dye. Recently, it was reported that most of cancers have an acidic microenvironment (acidic extracellular fluid: pH 6.5-7.0) and numerous large lysosomes with strongly acidic vesicular fluid (pH 3.0-5.0) [16]. We demonstrated that high-grade malignant sarcomas have more acidic microenvironments than benign tumors and normal soft tissues [17]. Therefore, we could easily expect AO to selectively accumulate in sarcomas and be useful for detecting tumor localization in photodynamic diagnosis (PDD) during surgery with fluorescence [18]. If it were clinically applicable, we could detect fluorescing tumor tissue by fluorescence surgical microscopy (fluorovisualization effect), and precisely resect only tumor tissue. It causes minimal damage to normal tissues like major nerves, vessels, muscles, bones and joints, etc. [4]. It may be better to call such treatment photodynamic surgery (PDS). We also demonstrated that AO exerts selective cytotoxic effects against sarcoma cells both *in vitro* and *in vivo* after illumination with visible light or irradiation of low-dose X-rays. It is available clinically for photodynamic therapy (PDT) [16-21] or radiodynamic therapy (RDT) [22-23].

Based on the data of basic research, we employed reduction surgery supported by AO therapy (AOT) following local administration of AO for patients with musculoskeletal sarcomas. This phototherapeutic modality consists of three main procedures: 1) intraoperative PDS (iPDS) immediately after intraoperative PDD (iPDD), 2) intraoperative PDT (iPDT) after intra-lesional or extra-capsular marginal tumor resection, and 3) RDT immediately following surgery (postoperative RDT). We have obtained good local control rates and markedly better limb functions with this approach as compared to the results of conventional wide resection surgery which commonly causes serious limb dysfunction [24-31].

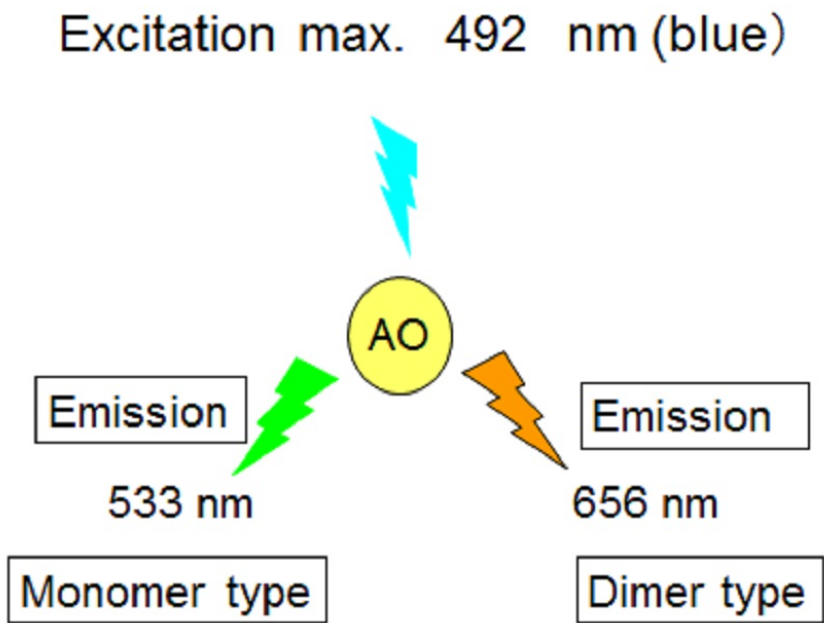
In the present review, we focus on iPDD and iPDS with local administration of AO using a fluorescence surgical microscope, demonstrating the results of basic research and details of the procedure of AOT for clinical application.

## Review

### Basic research

#### *Stainability of AO in Living Cells Vs. Apoptotic or Fixed Cells*

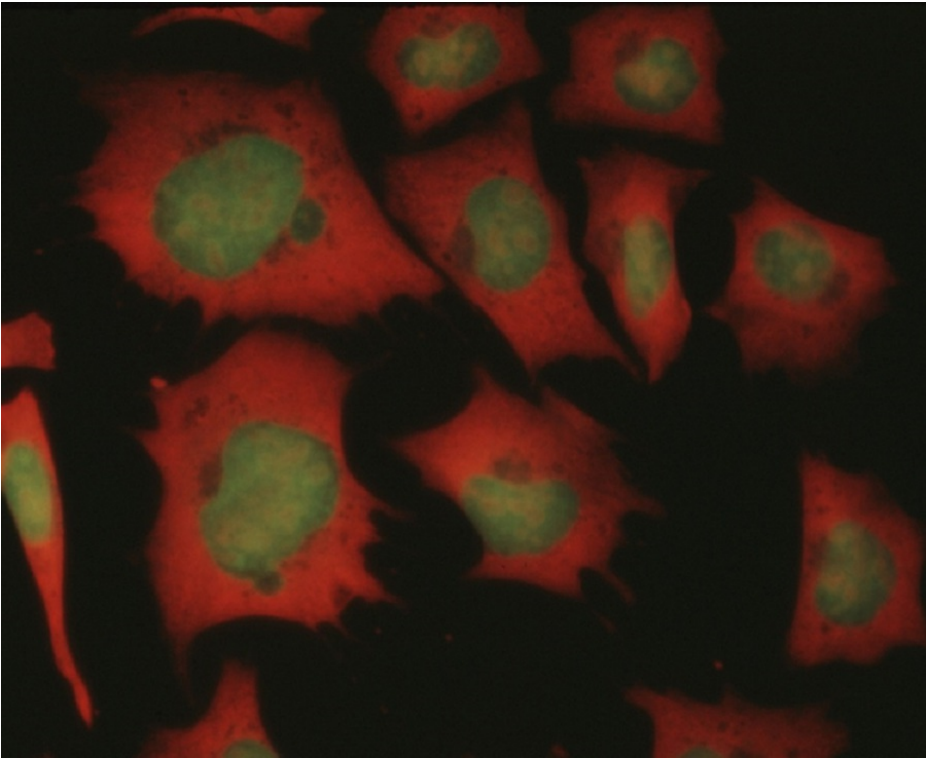
AO stains nuclear DNA as green fluorescence (533 nm), and cytoplasmic and nucleolar RNA, as red fluorescence (656 nm), after blue light excitation (492 nm) of cells fixed with ethanol followed by treatment with a two-step procedure using special phosphate buffer solutions [32-33] (Figures 2, 3). On the other hand, AO stains cytoplasmic and nucleolar RNA green and vesicles red in living cells in culture medium (Figure 4) [15].



**FIGURE 2: Excitation and emission (metachromasia) of AO**

AO is excited by blue light of 492 nm (max.) and emits green fluorescence (533 nm max.) from monomer type and red fluorescence (565 nm max.) from dimer type.

AO is excited by blue light of 492 nm (max.) and emits green fluorescence (533 nm max.) from the monomer type and red fluorescence (565 nm max.) from the dimer type.

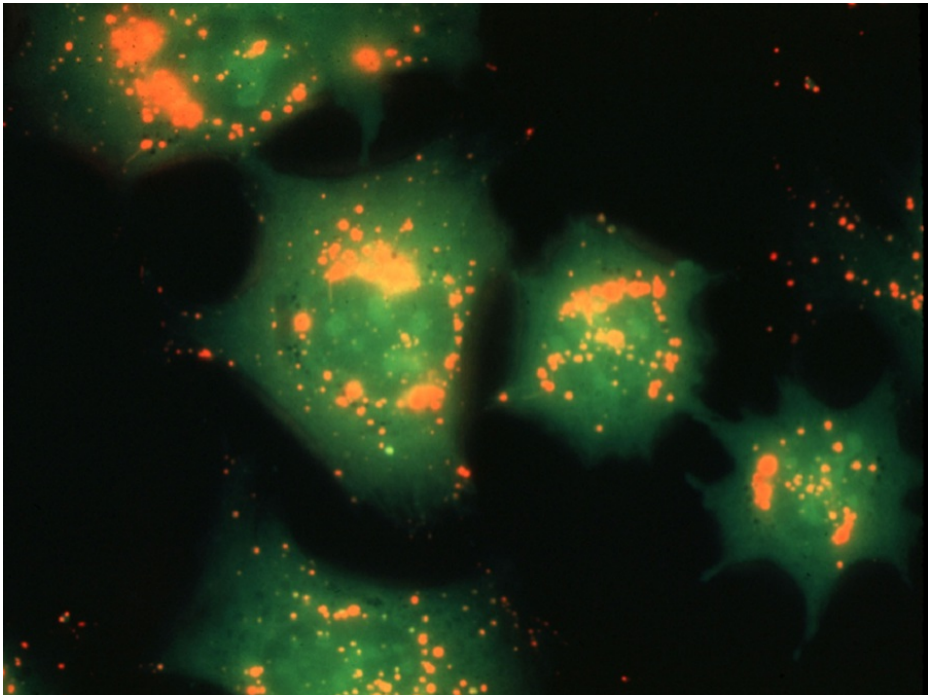


**FIGURE 3: Fluorescence view of mouse osteosarcoma cells (MOS) stained with AO after ethanol fixation using fluorescence microscope**

From cytoplasm and nucleolus, AO densely binding to RNAs emits red fluorescence, while AO sparsely binding DNA from nucleus emits green fluorescence. Binding mechanism of AO to nucleic acid is

intercalation to their strands.

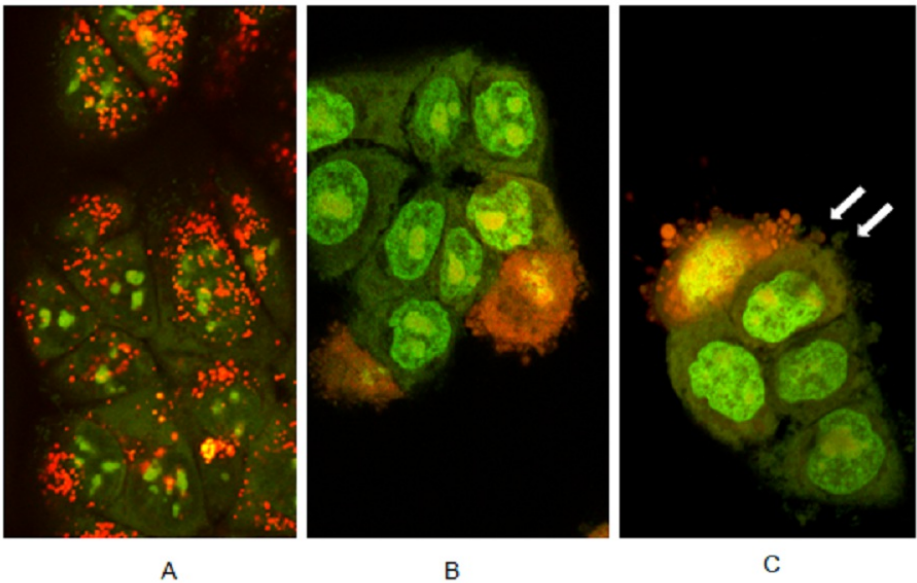
From cytoplasm and nucleolus, AO densely binding to RNAs emits red fluorescence, while AO sparsely binding DNA from nucleus emits green fluorescence. The binding mechanism of AO to nucleic acid is intercalation to their strands.



**FIGURE 4: Fluorescence view of living mouse osteosarcoma cells (MOS) exposed to AO after excitation using fluorescence microscope**

AO binding to cytoplasmic and nucleolar RNAs emits green fluorescence, while acidic vesicles like lysosomes, emits red fluorescence.

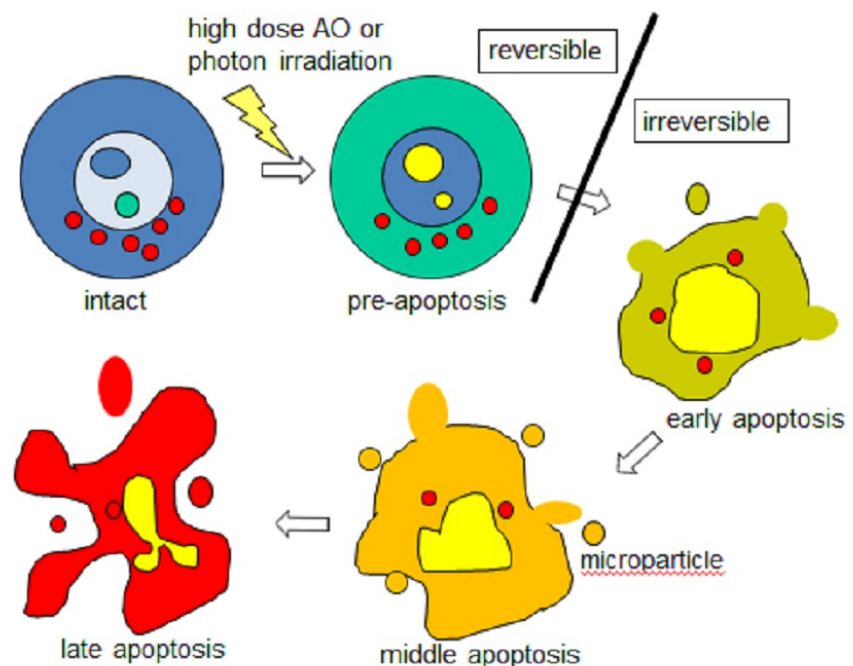
AO binding to cytoplasmic and nucleolar RNAs emits green fluorescence, while acidic vesicles like lysosomes emits red fluorescence. Nuclear DNA of intact living cells is not stained by AO, whereas that of apoptotic cells is stained (Figures 5, 6).



**FIGURE 5: Scanning fluorescence microscopic view of human gastric**

### cancer cells (MK28) exposed to AO after blue light excitation using a confocal laser fluorescence microscope

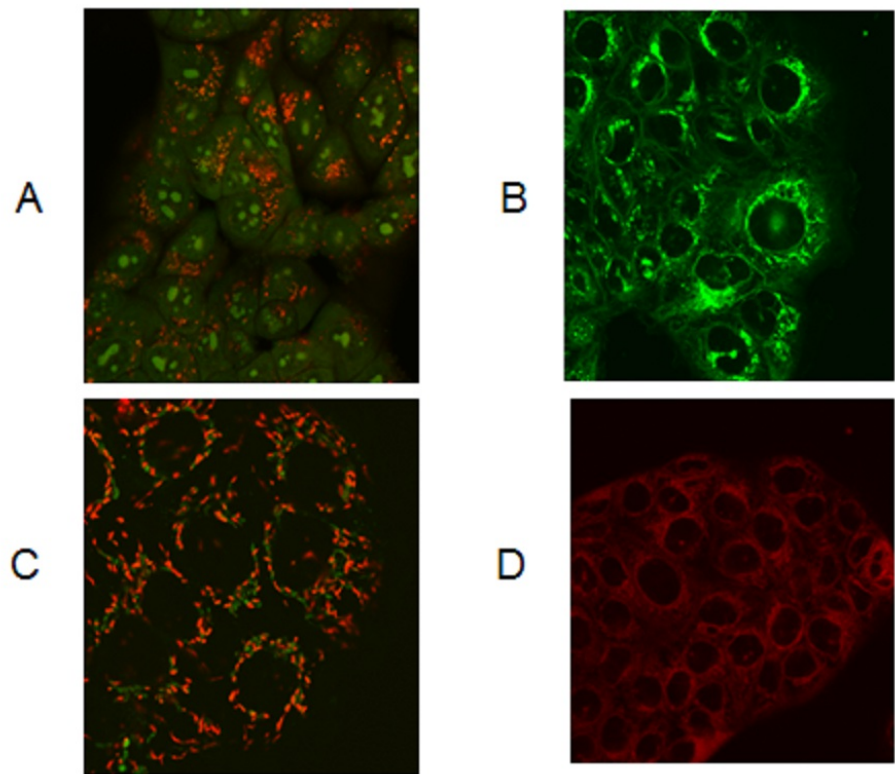
A: intact living cells, B: early and late apoptotic cells, C: blebs and microparticles of apoptotic cells (arrows)



**FIGURE 6: Sequential changes of AO stainability from intact living cells to apoptotic cells (see text)**

Thus, AO stainability differs completely between fixed cells and cultured cells. The different AO stainability of fixed and cultured cells has confused many for a long time. Since AO is a weak basic dye, it is ionized by protonation. AO is more soluble in acidic solutions, like HCl, than in alkaline solutions (AO powder is not soluble in concentrated NaOH solution). AO more easily accumulates in acidic environments of tissues than in neutral or alkaline environments. In fixed cells, AO binds more densely by intercalation to the acidic portions of single strands of RNA than to the double strands of DNA [6-7, 32-35]. Therefore, the dimer type of AO (dense AO) in the RNA emits red fluorescence, while the monomer type of AO (sparse AO) in the DNA emits green fluorescence. This phenomenon is the so called "metachromasia". On the other hand, in intact living cells, AO binds sparsely by intercalation to the acidic portions of transfer, micro, ribosomal, and messenger RNAs in the cytoplasm, emitting green fluorescence after blue light excitation. But AO does not bind to the double strands of nuclear DNA. AO shows dense accumulation in acidic vesicles, of which most are lysosomes, emitting red fluorescence. Intact living cells have intact bio-membranes, including cytoplasmic, nuclear and lysosomal membranes. This is the most important reason for the different stainability of AO between fixed cells, apoptotic cells and intact cells. Ionized AO cannot pass through such biological membranes because of the lack of special transporters or ion channels, whereas non-ionized AO can diffuse passively across these membranes [36]. Fixed cells, or apoptotic and dead cells have lost the membrane barrier system; therefore, AO binds to all acidic portions, independent of the biological proton distribution. The lysosomes of these cells have also lost their acidic fluid through the damaged membrane; therefore, AO does not accumulate in the lysosomes of these cells. In living cells cultured in neutral pH (7.4) medium, non-ionized AO quickly and passively enters the cytoplasm by diffusion through the cytoplasmic membrane containing numerous fatty acids and cholesterol for which AO shows strong binding affinity. AO rapidly binds to transfer RNAs and micro RNAs in the cytoplasm, as well as to the ribosomal RNAs and messenger RNAs in the nucleolus by intercalation. AO also accumulates in the acidic luminal fluid of lysosomes based on its affinity for protons. Since AO-binding RNAs emit green fluorescence, AO sparsely binds to the RNA in monomer form. AO in lysosomes is ionized well by protonation due to its strong acidity. Ionized AO cannot pass across the lysosomal membrane into cytoplasm for the same reason as that mentioned above. The concentration of ionized AO increases and ionized AO forms dimers under the high concentration of protons, which results in the emission by AO of red fluorescence following blue light excitation [36]. We have already morphologically confirmed that AO does not accumulate in other organelles, such as the mitochondria, Golgi apparatus, or endoplasmic reticulum (ER) (Figure 7) [15].





**FIGURE 7: Scanning fluorescence microscopic view of human gastric cancer cells**

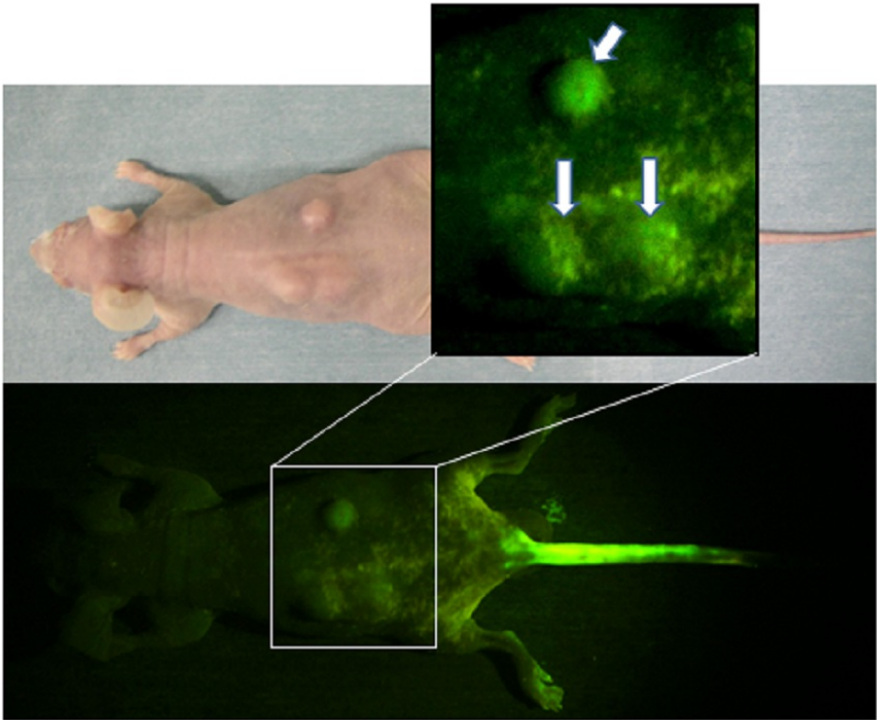
Scanning fluorescence microscopic view of human gastric cancer cells (MK28): (A) exposed to AO, (B) Golgi (BODIPY® FL C5-Ceramide), (C) mitochondria (JC-1), and (D) ER (ER tracker red)

AO does not bind to the nuclear DNAs in intact living cells, whereas it does bind to the nuclear DNA in apoptotic or dead cells (Figures 5, 6). This is the reason why AO is commonly used to detect apoptosis [37-38]. It is concluded that the binding targets of AO in intact living cells are the various RNAs and acidic vesicles, most of which are lysosomes.

#### *Selective Accumulation of AO in Mouse Osteosarcoma Cells In Vitro and In Vivo*

The results of our basic studies using mouse osteosarcoma cells (MOS cell line) have revealed that AO binds densely in dimer form to lysosomes and other acidic vesicles, including endosomes, phagosomes, secretion granules, etc., emitting orange fluorescence after blue light excitation. AO also binds only sparsely in the monomer form to the RNAs in the cytoplasm (transfer RNAs and micro RNAs) and nucleolus (ribosomal RNAs messenger RNAs), emitting green fluorescence (Figure 4).

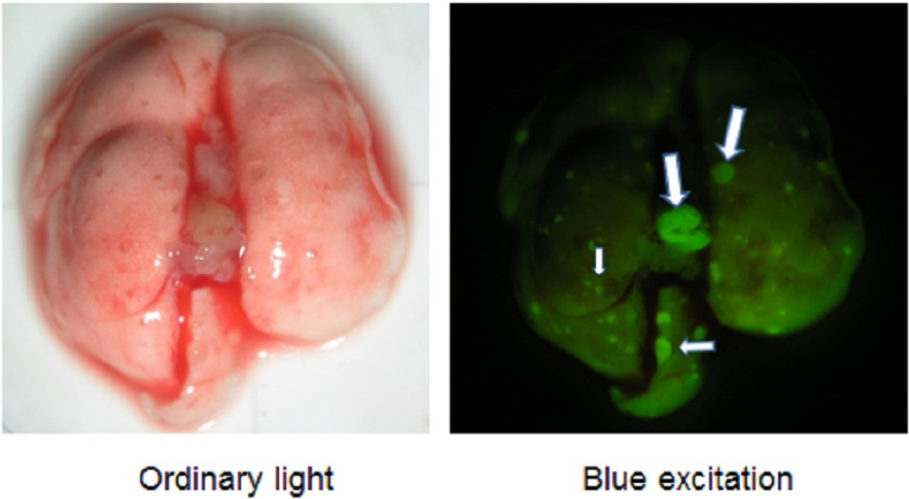
In an *in-vivo* study conducted using a mouse osteosarcoma model, the tumors emitted strong green fluorescence at two hours after intraperitoneal (10 mg/kg) or intravenous injection (1 mg/kg) of AO followed by blue light excitation, while normal muscle and adipose tissue cells did not emit any such fluorescence. Therefore, the tumors could be clearly visualized by fluorescence under the fluorescence surgical microscope equipped with a high resolution CCD camera (fluorovisualization effect) (Figure 8) [18, 20-21].



**FIGURE 8: Macroscopic fluorescence view of mouse osteosarcoma subcutaneously inoculated in the back of nude mouse after blue light excitation at two hours after AO injection through the tail vein (see text)**

Tumors emit green fluorescence (arrows). Injection site of the tail vein also emits green fluorescence.

Even small lesions, such as multiple pulmonary metastases from mouse osteosarcoma, measuring less than 1 mm of diameter can be easily detected by the emitted fluorescence (Figure 9).



**FIGURE 9: Macroscopic fluorescence view of pulmonary metastatic lesions of mouse osteosarcoma excised from same nude mouse as that shown in Fig. 8 (see text)**

Multiple metastatic lesions, even small lesions, emit brilliant green fluorescence (arrows).

We investigated the sequential changes of the AO fluorescence intensity from both mouse osteosarcomas and muscles after AO injection. The AO flowed rapidly into both the tumor and muscles; however, the muscles

excluded the AO quickly within two hours. On the other hand, the tumor excluded AO more slowly, the dye remaining in the tumor, even after two hours. Therefore, AO is retained for longer periods of time in the tumors than in the muscles [18, 21].

#### *Selective Accumulation of AO in Human Musculoskeletal Sarcomas Following Local Administration*

The surgically resected and cut tumor specimens without a tumor capsule emit intense green fluorescence from only the tumor tissue, and not from the surrounding normal tissues, after exposure to AO solution followed by blue light excitation. We confirmed that most human malignant bone and soft tissue tumors are sensitive to AO [17].

Using fresh and cut sarcoma specimens, we investigated the relationship between tumor acidity and the AO fluorescence intensity. The average pH of resected tissues measured using a needle-type pH meter was 6.78 in 35 sarcomas, 7.16 in 27 benign tumors, 7.26 in normal muscles, and 7.43 in normal adipose tissues. The fluorescence intensity of AO increased in a manner dependent on the acidity of these tissues. The acidic malignant tumors showed strong AO fluorescence. These results suggest that selective AO binding to musculoskeletal sarcomas is due to the acidic extracellular fluid and acidic lysosomes of the sarcoma cells [17]. Staining with AO is therefore useful to visually detect residual tumor tissues during surgery after tumor curettage under a fluorescence surgical microscope. It makes easy for the surgeons to additionally and completely excise only tumor tissue while causing minimal damage to normal tissues, as iPDS [17-18].

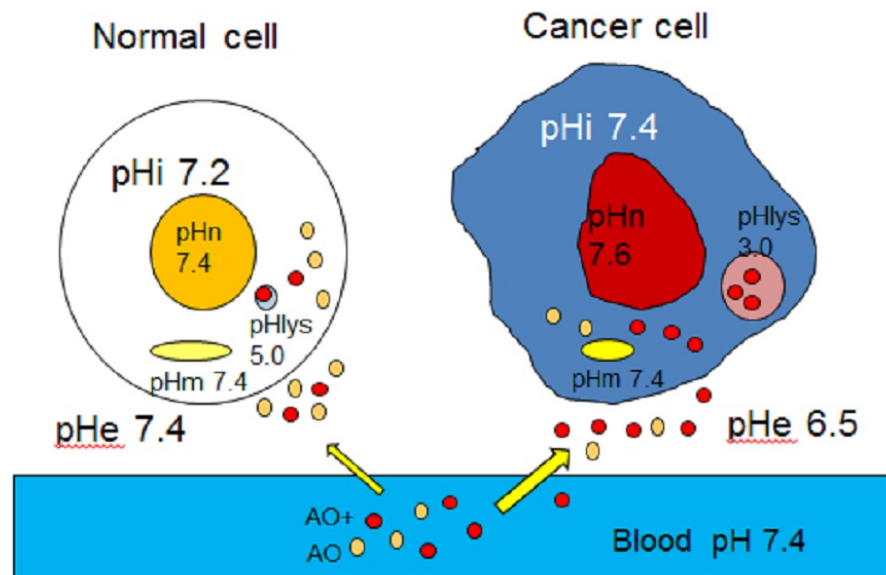
#### *Mechanism of Selective AO Accumulation in Sarcomas*

Although AO has been shown to stain all living cells, including not only sarcoma cells, but also normal cells in an *in-vitro* study, AO accumulates only in sarcomas and emits green fluorescence with blue light excitation at two hours after systemic AO administration. We also showed in an *in vivo* research that AO accumulated in all cells, but normal cells excluded AO more rapidly than sarcoma cells. However, we did not have any evidence to suggest that normal cells (fibroblast cell line) excluded AO more rapidly *in vitro*, although the AO fluorescence intensity and cytotoxic effect of AO-PDT was weaker in fibroblasts than in sarcoma cells. This phenomenon suggests the existence of some difference in AO accumulation in normal and sarcoma cells between *in vitro* and *in vivo* conditions.

Many recent studies have revealed that 1) cancer cells produce numerous protons ( $H^+$ ) by active glycolysis under hypoxia, or the Warburg effect [39-40]; 2) the protons are stored in lysosomes and other acidic vesicles by vacuolar-type  $H^+$ -ATPase (V-ATPase), resulting in the lysosomes and other acidic vesicles becoming even more acidic in the cancer cells than in normal cells [41-42]; 3) the extracellular fluid around cancer cells is also more acidic due to the larger number of protons excluded from the cytosolic space by NHE ( $Na^+ - H^+$  exchanger), V-ATPase and MCT (monocarboxylate transporter) than that of normal cells, or the larger number of protons produced by carbonic anhydrase 9 [43]. Since AO accumulates in acidic environments, sarcoma cells with a large number of acidic vesicles, are not able to exclude AO easily, whereas normal cells with non-acidic environments and weak acidic lysosomes can quickly exclude AO. It has been clarified that inhibition of V-ATPase activity by bafilomycin causes a decrease in AO accumulation in the lysosomes [44]. It suggests that AO accumulates in lysosomes in an acidity-dependent manner.

On the other hand, the pH partition theory [45] hypothesizes that weak basic agents like AO or many anticancer drugs, such as doxorubicin, accumulate in acidic fluids of the human body and are ionized by protonation (Figure 10).



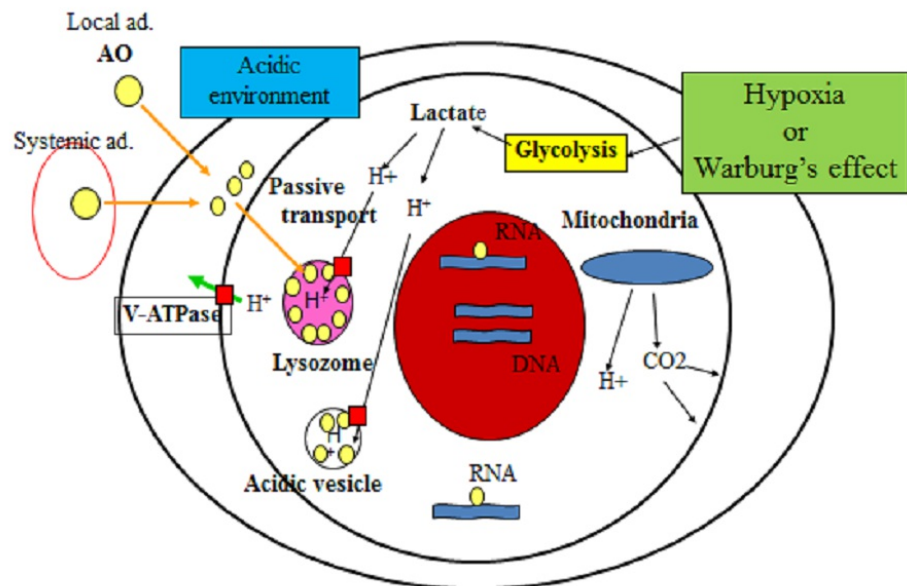


**FIGURE 10: AO accumulation mechanism speculated by the pH partition theory in normal and cancer cells in vivo**

Non-ionized AO (yellow) first accumulates in acidic extracellular fluid, with some becoming ionized (red) by protonation. Ionized AO cannot enter the cytoplasm, while non-ionized AO can easily enter the cell by diffusion. Non-ionized AO in the cytoplasm can also accumulate in the lysosomes by diffusion, depending on the acidity and ionization level in the lysosomes; ionized AO cannot diffuse back into cytoplasm because of the membrane barrier.

Non-ionized AO (yellow) first accumulates in acidic extracellular fluid, with some becoming ionized (red) by protonation. Ionized AO cannot enter the cytoplasm, while non-ionized AO can easily enter the cell by diffusion. Non-ionized AO in the cytoplasm can also accumulate in the lysosomes by diffusion, depending on the acidity and ionization level in the lysosomes. Ionized AO cannot diffuse back into cytoplasm because of the membrane barrier.

Therefore, our hypothesis to explain the AO accumulation mechanism in sarcoma cells is as follows. Active glycolysis through lactate produces a large number of hydrogen ions (protons) that are stored in the lysosomes or acidic vesicles by V-ATPase. The extracellular fluid around the cancer cells is also acidic due to the extrusion of protons by various proton transporters. Locally or systemically administered AO first accumulates in the acidic extracellular fluid and passively diffuses into the cytosolic space followed by accumulation in lysosomes based on the low pH (high acidity). Cytoplasmic AO also binds sparsely to RNAs by intercalation, which is a different type of binding to that in the lysosomes (Figure 11).



**FIGURE 11: AO accumulation in the extracellular space, cytoplasm, RNAs and lysosomes (see text)**

#### AO-PDT and AO-RDT

We also found that AO exerted a strong cytotoxic effect on mouse osteosarcoma cells after blue light illumination (AO-PDT), both *in vitro* and *in vivo*. *In vitro*, the osteosarcoma cells died quickly within 24 hours after exposure to 1 µg/ml of AO followed by light illumination for 10 minutes with 10,000 luminescence (same as lux units), and all of the cells died within 72 hours. This cytotoxic effect was even noted in multi-drug resistant osteosarcoma cells. In an *in-vivo* study carried out using a mouse osteosarcoma model, tumor growth was significantly inhibited by AO injection at 10 mg/kg into the peritoneum followed by light illumination of the tumor. This result suggested the potential usefulness of AO for photodynamic therapy in patients with musculoskeletal sarcomas [20-22].

Furthermore, we found that low-dose X-ray irradiation at 5 Gy after exposure to AO of a mouse osteosarcoma yielded the same strong cytotoxic effect *in vitro* and *in vivo* as that of by AO-PDT [22]. This radiation effect with AO was independently demonstrated by an Iowa University group in the USA in 2006 [23]. They showed that AO exposure followed by X-ray irradiation at 3 Gy significantly enhanced the cell death of radio-resistant chondrosarcoma cells, but not of the radio-sensitive cells. Therefore, this effect might be mediated through a different mechanism from that of AO-PDT. However, AO definitely enhanced the radiation-induced killing of cancer cells. We have named the treatment method based on this phenomenon radiodynamic therapy (RDT). X-ray irradiation has the advantage of reaching deeper areas of the human body than a light beam, even though it is more injurious to normal tissues. AO invades deeper tissues quickly at the rate of 5 mm per 30 minutes.

The schema in Figure 12 shows the mechanism of lysosomes by AO-PDT. AO accumulates in the lysosomes of cancer cells in an acidity-dependent manner. With the photon energy of blue light illumination, AO is excited by energy transfer. The excited AO activates the oxygen in the lysosomes, while emitting fluorescence, to become a stable type of AO. Activated oxygen (singlet oxygen) oxidizes the fatty acids of the lysosomal membranes, resulting in leakage of various lysosomal enzymes, such as proteases, lipases, and nucleases, into the cytosolic space. It has been reported that singlet oxygen scavengers, like L-histidine, inhibited the cytotoxic effect of AO-PDT [19, 36].

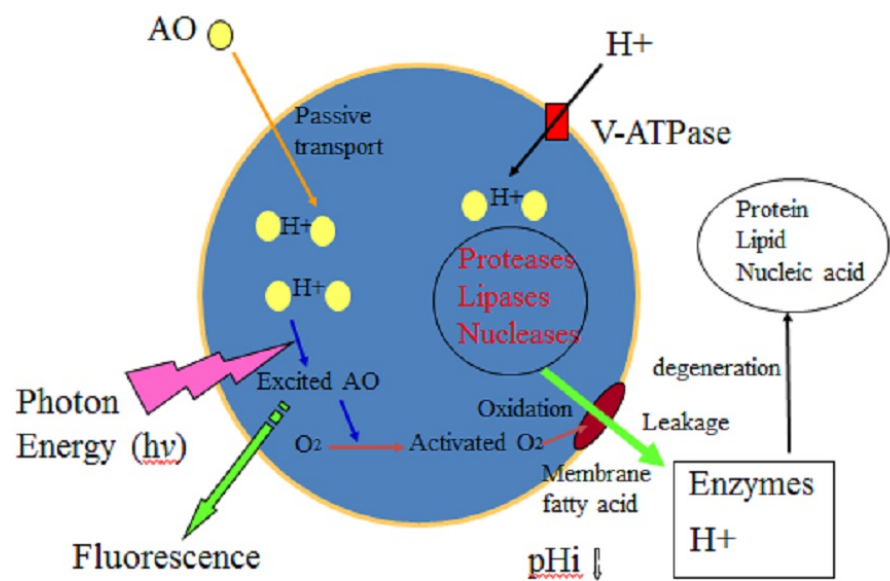


FIGURE 12: Process of lysosomal destruction by AO-PDT (see text)

A large amount of protons also leaks into the cytosolic space, which then becomes acidic. These enzymes digest important cell components under the acidic cytosolic condition. Such lysosomal stress activates caspase, which induces cellular apoptosis with cell swelling, bleb formation, and release of microparticles, including exosomes, eventually followed by cellular fragmentation (Figures 5, 6).

Clinical application of AOT

Procedure of AOT for Patients with Musculoskeletal Sarcomas (Figure 13A-13E).

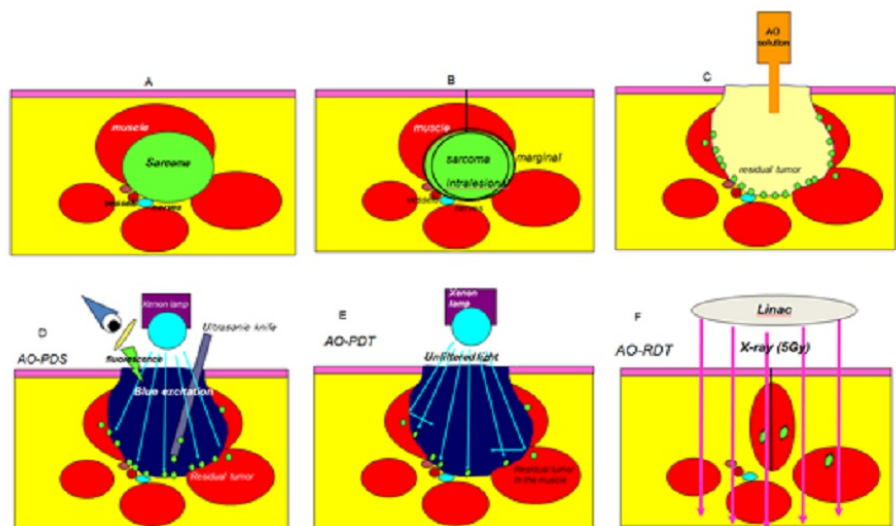


FIGURE 13: Procedure of AO therapy for clinical application (see text)

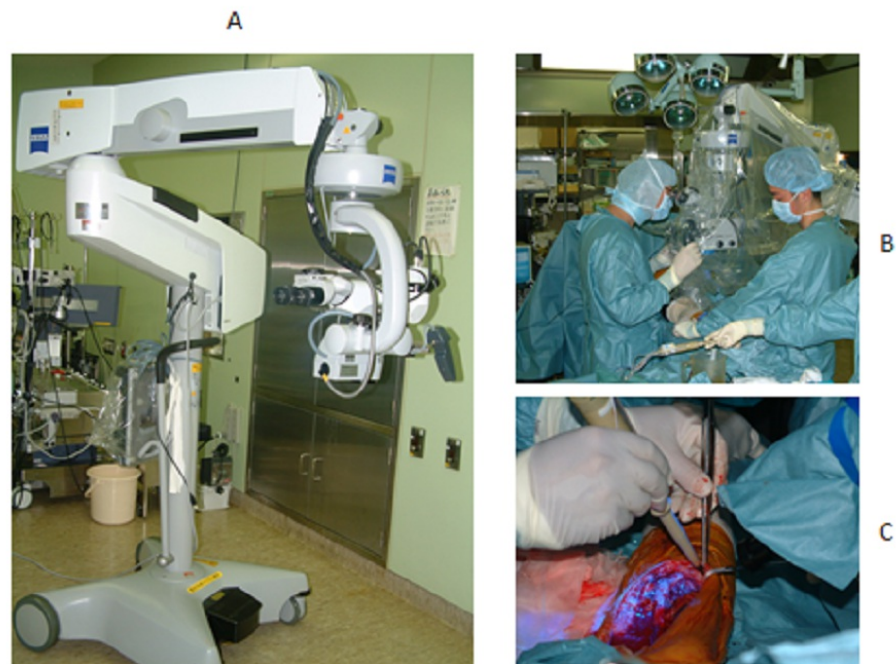
A) Sarcoma in the muscle close to major vessels and nerves; B) Tumor resection with marginal / intralesional margin; C) Local administration of AO solution D) Additional tumor resection under a fluorescence microscope; (Photodynamic Surgery: AO-PDS) E) Photodynamic Therapy (AO-PDT); F) Radiodynamic Therapy (AO-RDT)

1) Macroscopic curettage of tumor (Figure 13A, 13B)

If a sarcoma is localized in the muscle or bone close to major nerves, vessels, muscles, or bones and joints, intralesional tumor excision or marginal resection (extra-capsular resection) of the tumor with partial curettage is performed. These procedures are performed to minimize the damage to intact muscles and bones or to the major nerves and vessels in close contact with the tumor, in order to obtain good limb function after surgery.

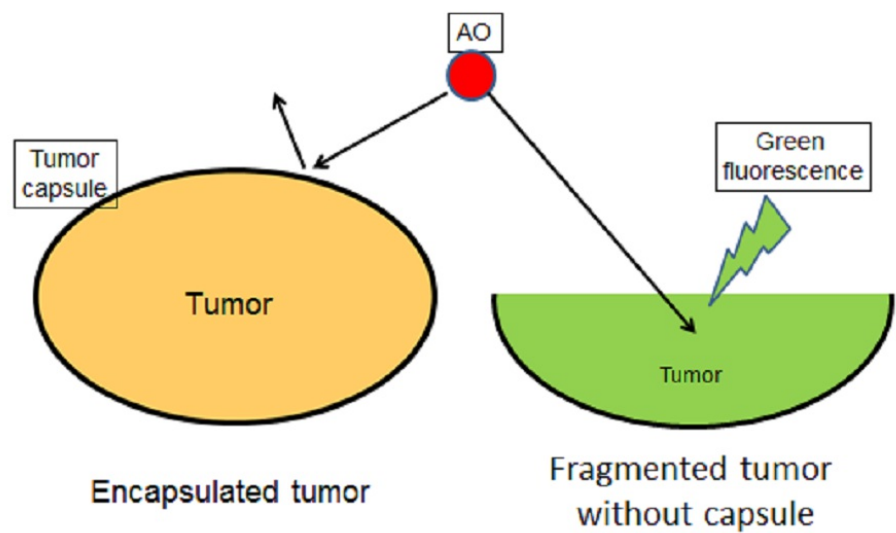
## 2) Intraoperative Photodynamic Surgery (iPDS) with Local Administration of AO (Figure 13C)

Microscopic curettage with an ultrasonic surgical knife (Olympus Co. Ltd., Tokyo, Japan) is additionally performed using a fluorescence surgical microscope. After local administration (soaking) of 1  $\mu\text{g}/\text{ml}$  of AO solution (SIGMA-ALDRICH CO, St. Louis, MO, USA) for five minutes, washing out of the excess AO solution with saline, and excitation with blue light is followed for fluorovisualization of the tumor. The surgical microscope manufactured by Carl Zeiss Co., Ltd, Oberkochen, Germany is specially equipped with an interference filter (466.5 nm) for selection of the blue beam from a xenon lamp and an absorption filter (>520 nm) for observation of the green fluorescence of AO (Figure 14A). The iPDS was repeated until complete disappearance of the green fluorescence from the remnant tumor tissue (Figure 14B, 14C).



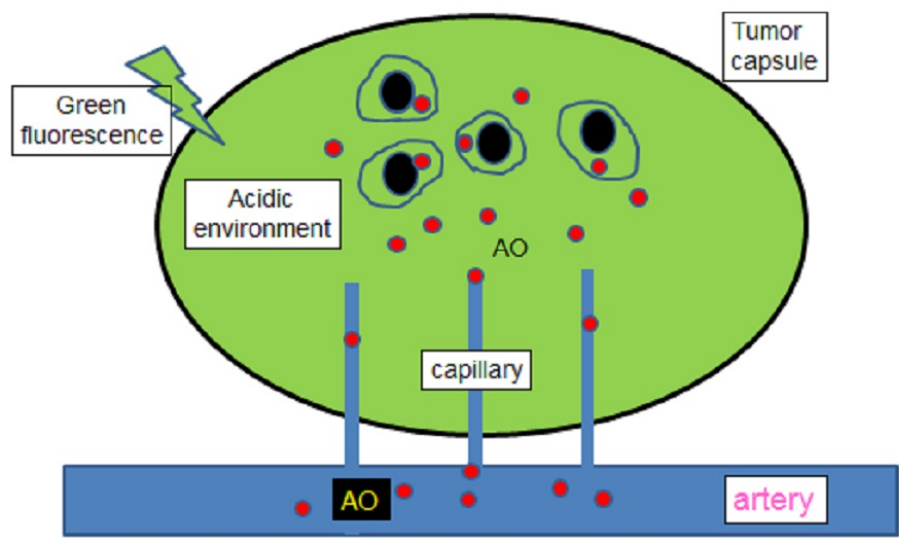
**FIGURE 14: Fluorescence surgical microscope (A) and scenes of PDS during surgery (B and C)**

In this procedure, we apply AO locally by soaking to expose residual tumor fragment after macroscopic tumor excision. If the same procedure was applied to the encapsulated tumor before excision, AO does not effectively accumulate in the tumor tissue, including in the interstitial stroma or cells, because the tumor capsule serves as a barrier between the non-acidic normal and acidic cancer extracellular fluid (Figure 15).



**FIGURE 15: Difference in AO accumulation following local administration between encapsulated and fragmented (non-encapsulated) tumor tissues (see text)**

Therefore, for local administration of AO, macroscopic tumor curettage to remove capsular barrier is a prerequisite. However, if AO is administered systemically, such as by intravenous injection two hours before the surgery, AO effectively and homogeneously accumulates even in the encapsulated tumor because it diffuses into the acidic stroma and tumor cells via the capillaries (Figure 16).



**FIGURE 16: AO accumulation in encapsulated tumor tissue by systemic administration (see text)**

Although we have never tried intravenous administration of AO in humans, no serious adverse effects were observed following administration of AO at the dose of 1  $\mu\text{g}/\text{ml}$  to mice and dogs in an experimental study. In the near future, we propose to investigate the safety of intravenous administration of AO to humans by carrying out a clinical study with the approval of the IRB, since systemic administration of AO is much better



in PDD to detect multiple lesions, such as metastasis or dissemination than local administration.

### 3) Intraoperative Photodynamic Therapy (iPDT) (Figure 13E)

After iPDS, intraoperative AO-iPDT is sequentially applied to the tumor curettage area for 10 minutes using a surgical microscope under illumination with unfiltered light (more than 5000 lx) from a xenon lamp.

### 4) Postoperative Radiodynamic Therapy (RDT) (Figure 13F)

After closure of the surgical wound without washing out the AO solution, AO-RDT is applied to the resected area immediately by X-ray irradiation of 5 Gy in a single session in the radiotherapy room for AO-RDT [24, 29-31].

### Patients

The 67 patients with high-grade malignant musculoskeletal sarcomas received AOT under approval from the IRB of our hospitals. The treatment was undertaken with the consent of the patients and/or their close families obtained after a full explanation was provided about the method/purpose of the clinical study. The 51 soft tissue sarcoma patients included patients with high-grade malignant sarcomas, such as synovial sarcoma, rhabdomyosarcoma, MHFs, leiomyosarcoma, etc., and the 15 bone sarcoma patients also included patients with high-grade malignant sarcomas, such as osteosarcoma and Ewing's sarcoma.

### Clinical Outcome

Analysis of the clinical outcomes in our study showed that the five year survival rate (SR) was 67.8% and the five year local recurrence-free rate (LRFR) was 71.2% in the 57 patients with soft-tissue sarcoma. According to the AJCC classification, the SR was 100% for Stage II cases, 86.7% for Stage III cases and 0% for Stage IV cases, and the LRFR was 92.3% for Stage II cases, 64.3% for Stage III cases, and 60.2% for Stage IV cases. In regard to the results classified according to the tumor size, the LRFR was 78% for tumors than 10 cm in diameter, while it was only 46.2% for larger tumors (Figure 2). On the other hand, the SR was 85.7% and the LRFR was 93.8% in the 15 cases with high-grade malignant bone sarcoma (Figure 3). Thus, while the local control rate after AO-PDT may not be superior to that after conventional wide resection surgery, the residual limb function is far superior as compared to that after wide resection. Most of the patients enrolled in our study showed excellent limb function for running fast, jumping, swimming, and throwing a ball [31].

We believe that all of our patients enrolled in this study would spend the rest of their lives as nearly normal or at least not handicapped people. Maintenance of good limb function is very important for sarcoma survivors, especially children, in order to ensure a long life with a high quality of life after surgery.

## Toxicity and carcinogenicity of AO

It is generally assumed that because AO is mutagenic in bacteria [46-47], it would be carcinogenic in humans. However, that is not true. Several studies have been carried out to investigate the carcinogenicity of AO [48-49]; however, none has provided any evidence to suggest the carcinogenicity of AO. Therefore, the International Agency of Research on Cancer (IARC) of the WHO and other official reports do not classify AO as a carcinogen [50]. There are some reports in the literature, in addition to ours, on the application of AO to human clinical studies. Two Japanese papers have reported using local/oral administration of AO for the diagnosis of cervical cancer or gastric cancer [51, another in Japanese]. Recently, a study reported by an Italian group applied our method of AO-RDT to a patient with synovial sarcoma [52]. In 2009, a group from USA also applied AO to some patients with ovarian disease in a clinical study of confocal laser laparoscopic biopsy under FDA approval [53]. The FDA in the USA has provided approval for the application of AO to particular clinical studies after investigation of the acute and chronic toxicity and carcinogenicity of AO using mice [54]. Our study using mice revealed that the LD<sub>50</sub> of AO following intravenous administration was 28-30 mg/kg [18]. The toxicity of 0.1 µg/ml AO administered by intravenous injection was investigated in dogs and no significant adverse effects were recognized [55]. None of the above clinical or experimental reports have shown evidence of any serious complications caused by AO administration. Since the concentration of AO solution used by us in our present clinical study was very low, and the substance was administered only locally, we believe that the risk of carcinogenesis induced by AO in our patients was significantly lower than that associated with most other known anticancer agents.

## Conclusions

In conclusion, based on the results of basic research and clinical data, we strongly believe that AOT, consisting of AO-iPDS, AO-iPDT, and AO-RDT, may be a promising new limb salvage modality for excellent preservation of limb function with a low risk of local tumor recurrence in musculoskeletal sarcoma patients. This therapeutic modality may also be applicable to many other solid cancers, although studies on a larger number of patients with longer durations of follow-up are required to verify this notion.

In the AOT procedure, iPDS is useful to detect the residual tumor tissue after intra-lesional tumor excision and makes it easy to precisely carry out additional excision of residual tumor tissue under fluorovisualization using a fluorescence surgical microscope.

## Additional Information

### Disclosures

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