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Determination of the LD_{50} of Acridine Orange *via* Intravenous Administration in Mice in Preparation for Clinical Application to Cancer Therapy

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Abstract. *Aim: We undertook studies to determine the lethal dose 50 (LD_{50}) of acridine orange (AO) using mice in order to confirm the safety of intravenous administration of AO. Materials and Methods: We used 40 mice and AO was administered once intravenously. General behavior and mortality were continuously observed for 14 days. At the end of the experiment, all animals were sacrificed for subsequent studies. Results: The LD_{50} for AO in male and female mice was determined to be 32 mg/kg and 36 mg/kg, respectively. Histopathological abnormalities were observed in only one mouse which died three days after the administration of AO. The other nine mice which died immediately after the administration of AO had no pathological findings in major organs. Conclusion: The clinical use of AO can be kept at 1.0 mg/kg or below and, therefore, intravenous administration of AO might be safe for use as cancer therapy.*

We have been developing an innovative limb salvage surgery modality using acridine orange (AO) for patients with musculoskeletal sarcoma, aiming to preserve excellent limb function with a low risk of local recurrence after intra-lesional or marginal tumor excision (1-5). This new surgical modality consists of a three-step procedure: i) photodynamic surgery (PDS) which makes it possible to additionally excise residual tumor after primary tumor excision under observation of the fluorescence of AO, which selectively accumulates in the tumor tissue and emits green fluorescence following excitation with a blue light beam using a surgical fluorescence microscope; ii) photodynamic therapy (PDT), which kills only tumor cells incorporating AO by a photoactivation effect because the AO accumulated into acidic lysosomes activates oxygen which destroys the lysosomal membrane *via* the oxidation of fatty acids; and iii) radiodynamic therapy (RDT), by which AO enhances the anticancer effects of radiation with a low dose of X-rays (5 Gy in one fraction). In this procedure, after macroscopic local tumor excision with minimum damage to normal tissues, such as nerves, vessels, muscles, joints or bones, a 1 μg/ml solution of AO is locally administered into the surgical field for five minutes, and excessive AO solution is removed. AO-PDS is first performed, followed by AO-PDT and AO-RDT. The clinical outcome of this pilot study showed good local tumor control and excellent limb function. In the clinical study, there were no serious complications associated with the local administration of the 1 μg/ml AO solution, nor by the illumination of light, or radiation with X-rays (1-5).

In the next step of AO therapy, systemic administration of AO by intravenous injection would be beneficial because it is more effective to deliver AO to the whole body and homogenously expose all acidic cancer tissues to AO than to perform the local administration we have been performing. We also reported that AO was available for the photodynamic diagnosis (PDD) of osteosarcomas using a mouse model when AO was intravenously injected followed by blue light illumination (6). Therefore, if intravenous injection of AO could be safely applied to humans, it might also be possible to detect cancer by PDD and simultaneously selectively kill only cancer tissues by PDT and RDT with or without PDS, even if they are of small size or at an early stage, or if there were multiple lesions, such as multiple metastatic or locally disseminated lesions. This is an ideal cancer therapy and is associated with low costs.

Although it has already been reported that AO is mutagenic for bacteria, no evidence has been found that AO is carcinogenic in mammalians (7-9). In the present study, we therefore undertook studies to determine the LD_{50} (the concentration that kills half of the population, a value commonly used to assess toxicity) of AO using mice in order to confirm the safety of intravenous administration of AO.

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Materials and Methods

Animal study. To investigate the acute toxicity of AO in mice, we used 40 healthy Crlj:CD1(ICR) mice (5-week-old males and females, 26.6-31.6 g and 22.0-25.1 g in body weight, respectively; Japan Charles River Laboratories, Atsugi-city, Kanagawa, Japan) and divided them into eight groups of five mice each. AO (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was administered once intravenously at 21, 25, 30 or 36 mg/kg to each of the four groups for each gender. These doses were determined by referring to the data from our previous pilot study to estimate the rough value of the LD_{50}. AO was injected through the mouse tail vein. After the injection, the general behavior and mortality of the mice were continuously observed for 6 h after the administration of AO, and then daily thereafter, for a total of 14 days. The mice were weighed on days four, eight and 14. At the end of the experiment, all animals were sacrificed for subsequent experimental studies. The following organs and tissues were fixed by immersion in neutral-buffered 10% formalin: brain, heart, kidneys, liver, lungs, spleen, thyroid grand, ovaries, testes, stomach, duodenum, colon, urinary bladder, prostate, femur and thigh muscle. All samples were embedded in paraffin wax. Histological sections stained with hematoxylin and eosin were then prepared. We performed this study with an authorized standard method to detect acute toxicity using mice, with the cooperation of the Mitsubishi Chemical Corporation (Tokyo, Japan), which was approved as study number of B080071.

Statistical analysis. The statistical analyses were performed using the Stat View statistical software program, version 5.0 (SAS Institute Inc Cary, NC, USA). Significant differences among the groups were evaluated using a paired *t*-test. A value of $p<0.05$ was considered to be statistically significant. All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education.

Results

No obvious differences were found in the weight gain between the treated groups (Figure 1). None of the male or female mice receiving 21 mg/kg of AO developed any symptoms. All male and female mice receiving 25 mg/kg of AO developed symptoms such as ataxia, irregular respiration, falling down, inactivity and clonic convulsions for five minutes after administration, but they subsequently recovered and lived for the two study weeks prior to being sacrificed. Four male and one female mice receiving 30 mg/kg of AO developed symptoms mentioned followed by death. One male mouse that received 30 mg/kg of AO exhibited inactivity, a hunched position, hypothermia and lacrimation two days after administration of AO, followed by death the following day. Two each of the male and female mice that received 36 mg/kg of AO developed symptoms such as ataxia, irregular respiration, falling down, inactivity and clonic convulsions, followed by death. The remaining male and female mice that received 30 or 36 mg/kg developed such symptoms lasting for five minutes just after administration, but they recovered afterwards and lived for the two weeks until they were sacrificed. The LD_{50} for AO in male and female mice was therefore determined to be 32 mg/kg and 36 mg/kg, respectively (Tables I and II).

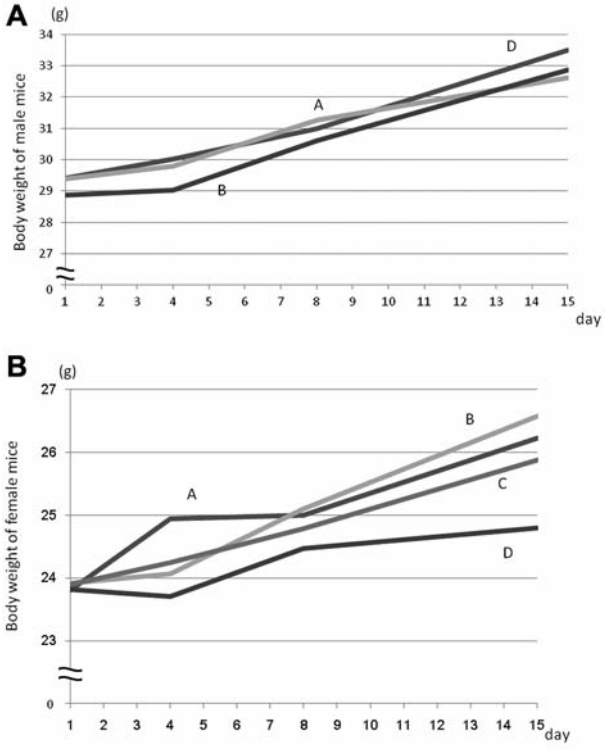

Figure 1. *There were no obvious differences in the weight gain between the different treatment groups. We excluded the mice which were dead within 14 days. Groups: A: 21 mg/kg, B: 25 mg/kg, C: 30 mg/kg, D: 36 mg/kg). Bodyweight of A: male mice; B: female mice.*

None of the major organs of the mice that died five minutes after the administration of AO showed any histopathological features suggestive of any specific toxicity. The male mouse which died three days after the administration of 30 mg/kg of AO showed edematous changes in the renal cells and bleeding in the mucous membranes of the urinary bladder, which suggested acute renal failure and hemorrhagic cystitis, respectively (Figure 2).

Discussion

For the clinical application of AO in cancer therapy, systemic administration by intravenous injection would more effective to deliver AO throughout the whole body and would homogenously expose acidic cancer tissues to AO compared to local administration, which we have been performing to date. We reported that AO was useful in detecting the localization of osteosarcomas inoculated into the subcutaneous soft tissues or in the bones of the extremities in mice by fluorescence observation with a high sensitivity charge-coupled device camera after intravenous AO injection *via* the tail vein,

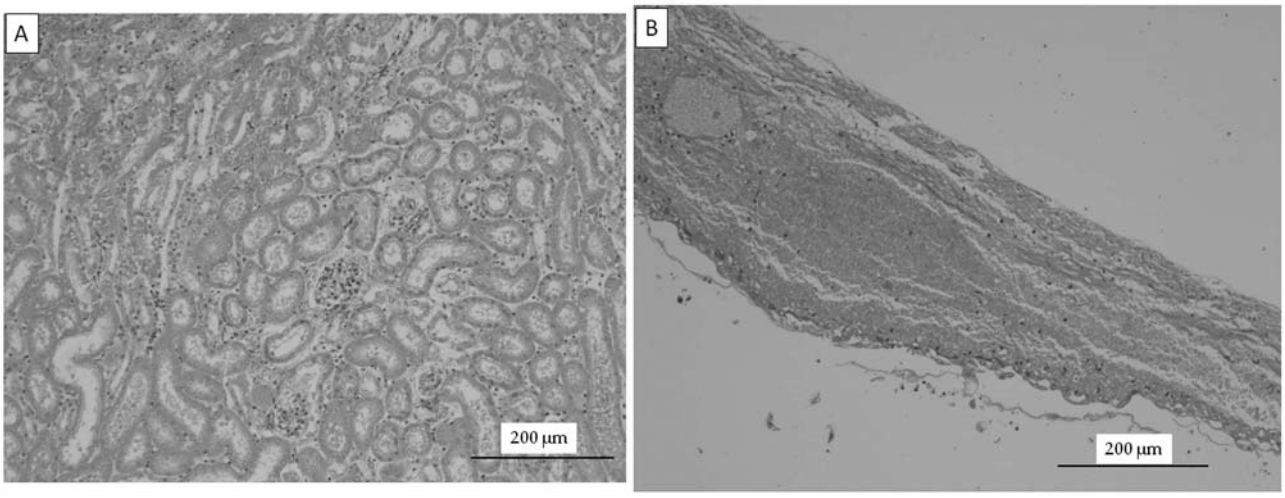

Figure 2. *There were edematous changes in the renal cells (A) and bleeding at the mucous membranes of the urinary bladder (B) of one male mouse that died three days after the administration of 30 mg/kg of AO, which suggested the development of acute renal failure and hemorrhagic cystitis, respectively (hematoxylin and eosin stain, ×10).*

Table I. *Cumulative mortality in mice after administration of AO.*

Gender	AO dose (mg/kg)	Number of mice	Day									Mortality
			1						2	3	4 to 15	(%)
			Pre	5 min	30 min	1 h	3 h	6 h				
Male	21	5	0	0	0	0	0	0	0	0	0	0
	25	5	0	0	0	0	0	0	0	0	0	0
	30	5	0	4	4	4	4	4	4	5	5	100
	36	5	0	2	2	2	2	2	2	2	2	40
Female	21	5	0	0	0	0	0	0	0	0	0	0
	25	5	0	0	0	0	0	0	0	0	0	0
	30	5	0	1	1	1	1	1	1	1	1	20
	36	5	0	2	2	2	2	2	2	2	2	40

followed by blue light illumination. Our previous study also revealed that the growth of these inoculated mouse osteosarcomas incorporating AO was significantly inhibited after illumination with a high-power flash wave xenon light (10). Furthermore, we showed that AO was useful in detecting pulmonary metastatic lesions in a mouse osteosarcoma model by monitoring the fluorescence, and AO had the ability to inhibit lung metastasis after intravenous injection (11). In these studies, we estimated that the LD_{50} was 27.3 mg/kg in C_3H mice (five-week-old males, 16.9-19.1 g body weight; Japan SLC Inc.) after performing the intravenous injection of AO at doses ranging from 0.1 mg/kg to 50 mg/kg (6). We used 1 mg/kg AO for the above studies because it provided the highest fluorescence contrast between the tumor and muscles surrounding the tumor.

The results of the present study showed that the LD_{50} for AO in male and female Crlj:CD1(ICR) mice was 32 mg/kg and 36 mg/kg, respectively. These doses are slightly higher than in our previous report (6). Although all male and female mice that received 25 mg/kg survived, they developed symptoms such as ataxia, irregular respiration, falling down, inactivity and clonic convulsions within five minutes of administration. Based on the present results, we conclude that a dose of ≤21 mg/kg is completely safe for mice because none of the mice that received 21 mg/kg showed any symptoms. Our previous study showed that there were no mice that died after the intravenous administration of AO at a dose less than 20 mg/kg (6). As mentioned above, 1 mg/kg of AO was sufficient to induce PDD, PDS and PDT in the mouse osteosarcoma model, and a higher concentration is not

Table II. *Clinical findings after administration of AO in mice*

Dose of AO (mg/kg)	Clinical findings	Day, no. of mice								
		1						2	3	4 to 15
		Pre	5 min	30 min	1 h	3 h	6 h			
Male mice										
21	No abnormality	5	5	5	5	5	5	5	5	5
25	No abnormality	5	0	5	5	5	5	5	5	5
	Lateral position	0	5	0	0	0	0	0	0	0
	Decreased locomotor activity	0	1	0	0	0	0	0	0	0
	Ataxic gait	0	1	0	0	0	0	0	0	0
	Irregular respiration	0	4	0	0	0	0	0	0	0
30	No abnormality	5	0	1	1	1	1	0	0	
	Death	0	4	0	0	0	0	0	1	
	Falling down	0	1	0	0	0	0	0	0	
	Hunckback position	0	0	0	0	0	0	1	0	
	Decreased locomotor activity	0	1	0	0	0	0	0	0	
	Clonic convulsion	0	5	0	0	0	0	0	0	
	Irregular respiration	0	5	0	0	0	0	0	0	
	Hypothermia	0	0	0	0	0	0	1	0	
	Lacrimation	0	0	0	0	0	0	1	0	
35	No abnormality	5	0	3	3	3	3	3	3	3
	Death	0	2	0	0	0	0	0	0	0
	Falling down	0	3	0	0	0	0	0	0	0
	Decreased locomotor activity	0	3	0	0	0	0	0	0	0
	Clonic convulsion	0	3	0	0	0	0	0	0	0
	Irregular respiration	0	4	0	0	0	0	0	0	0
Female mice										
21	No abnormality	5	5	5	5	5	5	5	5	5
25	No abnormality	5	0	5	5	5	5	5	5	5
	Falling down	0	4	0	0	0	0	0	0	0
	Decreased locomotor activity	0	4	0	0	0	0	0	0	0
	Clonic convulsion	0	3	0	0	0	0	0	0	0
	Irregular respiration	0	4	0	0	0	0	0	0	0
30	No abnormality	5	0	4	4	4	4	4	4	4
	Death	0	1	0	0	0	0	0	0	0
	Falling down	0	4	0	0	0	0	0	0	0
	Decreased locomotor activity	0	4	0	0	0	0	0	0	0
	Clonic convulsion	0	5	0	0	0	0	0	0	0
	Irregular respiration	0	5	0	0	0	0	0	0	0
35	No abnormality	5	0	3	3	3	3	3	3	3
	Death	0	2	0	0	0	0	0	0	0
	Falling down	0	3	0	0	0	0	0	0	0
	Decreased locomotor activity	0	3	0	0	0	0	0	0	0
	Clonic convulsion	0	3	0	0	0	0	0	0	0
	Irregular respiration	0	5	0	0	0	0	0	0	0

necessary because of the selective accumulation in the tumor compared to normal tissues. Our previous study using systemic administration by intraperitoneal injection of AO also revealed that the accumulation of AO in mouse osteosarcomas, as detected by the fluorescence intensity, was saturated at a dose of 10 mg/kg, which is predicted to be almost the same as that at 1 mg/kg by intravenous injection (12). Another study performed by a group of veterinarians using dogs showed that the administration of 0.1 mg/kg of AO by intravenous injection was safe, and did not induce any abnormalities in the blood examination, and did not lead to any serious symptoms. That study also revealed that the serum concentration of AO decreased rapidly within 30 min (13).

Researchers at the Arizona University reported a clinical trial of a confocal microlaparoscopic fluorescence-based diagnosis of human ovarian lesions including cancer using AO by intraperitoneal administration (14). Before the study, they observed that there was acute and chronic toxicity of AO (at

0.33 mM and 3.3 mM, respectively) following intraperitoneal injection in mice, and clarified the safety of its administration for clinical applications (15). The U.S. Food and Drug Administration (FDA) approved the use of AO in their clinical study.

The mechanism underlying the toxicity of AO remains unclear. In the present study, histopathological abnormalities were seen in only one mouse which died three days after the administration of AO. Although the other nine mice which died immediately after the administration of AO developed ataxia, irregular respiration, falling down, inactivity and clonic convulsions, there were no pathological findings in major organs such as the brain and heart. Although the cause of death could not be identified, the occurrence of rapid death without any degenerative changes in the organs suggest that it might have been caused by rapid hemolysis or damage to the central nervous system. We did not perform a blood or serum analysis in the present study, so further studies are required to elucidate the cause of death in the mice.

Even though the present study did not show any evidence of chronic toxicity, and no previous studies have found evidence of carcinogenicity for AO in mammals (16), insufficient studies have been performed to draw conclusions about its use in humans. However, AO is a known mutagen for bacteria. The International Agency for Research on Cancer of the World Health Organization reported that AO was considered to be not classifiable with regard to its carcinogenicity (class 3). No other official Institutions, such as the Physical and Theoretical Chemistry Laboratory of Oxford University, Pesticide Action Network Pesticides Database, or the FDA have classified AO as a carcinogen. Since 1999, we have used local AO administration to treat more than 100 patients with high-grade malignant musculoskeletal sarcomas, such as malignant fibrous histiocytoma, synovial sarcoma, rhabdomyosarcoma, osteosarcoma and Ewing's sarcoma, and none has suffered from secondary sarcoma or carcinoma at the surgical site (1-5). Therefore, we believe that AO is not carcinogenic in humans, at least not after a single application.

In conclusion, we clarified that the LD_{50} for AO in male and female Crlj:CD1(ICR) mice was 32 mg/kg and 36 mg/kg, respectively, based on the results of the present study. We believe that clinical use of AO can be kept at 1.0 mg/kg or below and, therefore, intravenous administration should be safe for humans for use as PDD and AO therapy.

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