

# Biodistribution of Three Photosensitizers in Dogs with Spontaneous Tumors\*

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## ■ ABSTRACT

Photodynamic therapy (PDT) has been considered a potential method for tumor eradication. The present study was designed to assess the efficacy of PDT as an alternative treatment approach. Photosensitizers, such as porfimer sodium, tin ethyl etiopurpurin, and aluminum chlorophthalocyanine, were administered IV to dogs, and tissue samples were harvested 24 to 300 hours later. The uptake of the photosensitizers in tumor (fibrosarcoma) and adjacent normal tissue biopsies was quantified by tissue solubilization technique and fluorimetry. In addition, the pharmacokinetics and selectivity of the photosensitizers were addressed by two-phase exponential function and specific uptake ratio, respectively. Porfimer sodium exhibited a longer elimination half-life (175.3 hr), slower clearance (0.0028 L/kg/hr), and a larger area under the curve (1075 µg/g/hr) in tumors than did tin ethyl etiopurpurin or aluminum chlorophthalocyanine. As a result, porfimer sodium showed a good selectivity in tumors located in muscle and skin.

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The study provided clinical information for determination of the efficacy of different PDT alternatives.

## ■ INTRODUCTION

Photodynamic therapy (PDT) involves the use of photosensitizers, which are most frequently administered by IV injection. Following a given time delay, photosensitizers are activated by light of an appropriate wavelength.<sup>1</sup> The only photosensitizer currently approved by the FDA for use in oncology is porfimer sodium. Administration of porfimer sodium is followed by illumination with laser light with a wavelength of 620 to 630 nm. Quantum yields were below 0.6 and adverse reactions (erythema and blistering) on the skin persisted for up to 8 weeks following treatment when the person was exposed to sunlight or bright indoor lighting.<sup>2</sup> Some of these effects are overcome by the so-called second-generation photosensitizers, such as tin ethyl etiopurpurin (SnET2) and aluminum chlorophthalocyanine (AlClPc) with chemical purity, minimal toxicity, and significant wavelength above 650 nm. These modifications may allow

light to reach deep into tissue with greater quantum yields and faster clearance from normal tissue.<sup>3</sup> These second-generation photosensitizers are currently being evaluated for clinical efficiency.<sup>4-6</sup> Ideally, the photoactivation of the drug will be initiated when the selectivity of photosensitizer is weighted toward the tumor, with minimal concentrations in normal tissue. Several studies have reported localization of photosensitizers in cell components after several hours of incubation and redistribution.<sup>7</sup> Therefore, knowledge of the pharmacokinetic properties of the photosensitizers to determine uptake in tumors and adjacent normal tissue is important for determining the optimum time for injection of the photosensitizer and maximum selectivity for PDT treatment.

To date, pharmacokinetic data have been obtained only from transplantable, implantable animal models, which are known not to reflect the heterogeneity in growth and physiology seen in humans tumors.<sup>8</sup> Only limited human biopsy data are available for pharmacokinetic analysis.<sup>9</sup> In the present study, animals with spontaneous tumors were used to determine the pharmacokinetic properties of three photosensitizers (porfimer sodium, SnET2, and AlClPc). The tumors and the surrounding normal tissue mimic human malignancies in terms of blood flow, oxygenation,

photosensitizer absorption, metabolism, and elimination.<sup>10-12</sup> Interanimal variability for photosensitizer concentrations in the animals would be similar to that in humans, and the model would provide useful data for the clinical application of PDT.

## ■ MATERIALS AND METHODS

### Chemicals

A commercial, ready-to-use solvent (Solvable; DuPont Biotechnology Systems) was mixed with all tissue samples. This solvent is a mixture of N,N-dimethyl laurylamine oxide 3%, aldyloxypolyethylene oxyethanol 3%, and sodium hydroxy 2%, which was developed for scintillation counting. Porfimer sodium, dissolved in 5% dextrose to a final concentration of 2 mg/ml, was supplied as a powder (Quadrologic Technologies). SnET2 (1 mg/ml) was provided in Cremophor EL emulsion (Miravant), and AlClPc (no sulfonation) was prepared in 5% dextrose (0.25 mg/ml). All three photosensitizers were administered IV.

### Animals and Tumor Model

Dogs with spontaneous tumors were recruited from veterinary clinics in southeast Michigan. Informed consent detailing different options for tumor cure, such as surgery or PDT, and surgical procedures were reviewed by the

**TABLE 1. Sampling Information for Dogs with Spontaneous Tumors Used for Evaluation of Three Photosensitizers**

Sample	Porfimer Sodium		SnET2		AlClPc	
	No. of Dogs	No. of Samples	No. of Dogs	No. of Samples	No. of Dogs	No. of Samples
Tumor	18	36	8	23	14	15
Skin	12	16	8	12	12	12
Muscle	14	14	8	13	ND	ND

AlClPc = aluminum chlorophthalocyanine; ND = not done; SnET2 = tin ethyl etiopurpurin.

local animal welfare committee. Twelve to 18 dogs, each weighing 24 to 32 kg, were used for each photosensitizer (Table 1). Dogs with spontaneous tumors present biologic behavior and patterns similar to those in humans, except for metastatic rate and growth, making them a suitable model for this evaluation. The tumors investigated were soft tissue sarcomas (fibrosarcoma) of the trunk or extremities. These tumors exhibit a predictable biologic behavior such as local invasion, with slow metastasis,<sup>13</sup> and hence were good candidates for a local therapy such as PDT. Surgical removal of the tumor, including limb amputation, was performed when anatomically feasible. Fibrosarcoma cells that had infiltrated along fascial planes of the tissue were difficult to distinguish on gross examination of the excised peripheral margins of the tumor. The dogs were randomly assigned to one of the three treatments (porfimer sodium [3 mg/kg], SnET2 [0.83 mg/kg], or AIClPc [0.209 mg/kg]).

Based on the extent of the tumor, dogs were scheduled for PDT treatment or limb amputation. Samples of the tumor and surrounding skin and muscle were removed for the pharmacokinetic evaluation prior to amputation (Table 1). For sample retrieval, the dogs were immobilized by general anesthesia (thiamylal sodium 18 mg/kg) for induction and intubation. Anesthesia was maintained by inhalation of isoflurane carried in oxygen. Atropine was administered as needed to regulate the heart rate. Heated circulating blankets were used to maintain the body at a constant temperature. Body temperature, heart rate, respiratory rate, and depth of anesthesia were monitored and recorded at 5-minute intervals. Tumor and adjacent normal tissue (muscle and skin) samples were removed surgically, frozen in liquid nitrogen, and kept at  $-80^{\circ}\text{C}$  in the dark until analysis. The amount of each tissue varied from dog to dog, and no attempt was made to

standardize the weight or dimensions of the tissues collected.

### Photosensitizer Quantification

The tissue solubilization method was used to measure photosensitization concentration in tissue samples. Photosensitizer concentration quantification methodology has been described extensively.<sup>14</sup> Prior to sample measurements, the fluorimeter (Photon Technologies Industries) was calibrated daily with a photobleaching-resistant, solid-state dysprosium:yttrium–aluminum–garnet (YAG) crystal. This procedure enabled absolute response calibration, correction of the source excitation, and transmission and emission of the monochromators involved. The stability of each photosensitizer (10 and 5  $\mu\text{g}/\text{ml}$ ) in the solvent was assessed to avoid chemical bleaching of the fluorescence properties over 6 hours. A window of 2 to 4 hours for tissue dissociation in the solvent was chosen as the optimal period for SnET2 and AIClPc quantification.<sup>14</sup> Fluorimeter responsivity ( $\zeta$ ), defined as detectable signal per unit of fluorophore concentration in the cuvette, was calculated by linear regression of the normalized signal as a function of known photosensitizer concentrations ranging from 10 to 0.01  $\mu\text{g}/\text{ml}$  over 2 hours.

Briefly, tissue samples (0.1 to 0.6 g each) collected from the dogs were divided into five fractions (extractions), minced, and immersed in 2 ml of solvent. The contents were placed in a 15-ml test tube, which was immersed in a shaking water bath at  $50^{\circ}\text{C}$  for 1 hour. The sample was then mechanically homogenized with a rotor/stator-type tissue homogenizer. Five aliquots of 200  $\mu\text{l}$  each were obtained and diluted in 3 ml of distilled water and 1 ml of solvent. The contents were returned to the warm bath for another hour. The optical density (OD) of each aliquot was measured in a glass cuvette with a 1-cm path using a double-

beam absorption spectrophotometer (Shimadzu Mandel Scientific). Depending on the tissue OD, samples were diluted with the volume of distilled water needed to achieve an OD less 0.1 for homogenous fluorescence excitation during optical quantification. Each aliquot had a different dilution rate depending on the animal, tissue sample, and extraction or fraction. The unknown concentration in the harvested tissue samples was calculated as follows:

$$C_t = \frac{M}{\zeta} \times \left( \frac{V}{V+2} + \frac{3+1+0.2}{0.2d} \right)^{-1}$$

where  $C_t$  is the concentration in the tissue,  $V$  is the volume of the solvent,  $M$  is the normalized fluorescence intensity,  $\zeta$  is the fluorimeter responsivity ( $\mu\text{g/g}$ ), and  $d$  is the dilution factor.

Porfimer sodium consists of a heterogeneous mixture of different porphyrins, and direct fluorescence intensity measurement similar to that used for SnET2 and AlClPc would have produced invalid data in aqueous solution.<sup>15</sup> Monomeric porfimer sodium compounds have higher fluorescence quantum yields than polymerized porfimer sodium compounds. It is possible to calculate the fluorescence intensity of porfimer sodium if the amount of monomer and the fluorescence of an equivalent standard

monomer compound are known. Up to five 200- $\mu\text{l}$  aliquots were collected, and five additional aliquots were each spiked with 0.05 ml of porfimer sodium (37.5  $\mu\text{g/ml}$ ). Depending on the tissue OD, samples were diluted either  $d_s$  (dilution of spiked sample) or  $d_{ns}$  (dilution of nonspiked sample) times in distilled water to achieve an OD less than 0.1. Finally, the aliquots were excited at 405 nm, and the fluorescence intensities were measured for all aliquots. The concentration in the original sample was given by:

$$C_t = \left[ \frac{M_s}{M_{ns}} \times \frac{d_s}{d_{ns}} - \frac{4.2}{4.25} \right] \times \left[ \frac{V+0.2}{V} \times \left( C_s \frac{0.05}{4.25} \times \frac{4.2}{0.2} \right) \right]$$

The results of up to five matched spiked and nonspiked aliquots were averaged to obtain the concentration of each tissue sample. For each tumor, up to five samples were prepared and analyzed.

### Analysis

The data collected for this experiment were subjected to two nonlinear regression (one-phase exponential [ $C_t = Ae^{-\beta t}$ ] and two-

**TABLE 2. Double Exponential Nonlinear Regression Coefficients for Different Photosensitizers Evaluated in Dogs with Spontaneous Tumors**

Sample	Porfimer Sodium		SnET2		AlClPc	
	Order	R <sup>2</sup>	Order	R <sup>2</sup>	Order	R <sup>2</sup>
Tumor	first	0.15	first	0.76	first	0.059
	second	0.15	second	0.76	second	0.059
Skin	first	0.43	first	0.78	first	0.56
	second	0.55	second	0.78	second	0.56
Muscle	first	0.42	first	0.69	ND	ND
	second	0.42	second	0.71	ND	ND

AlClPc = aluminum chlorophthalocyanine; ND = not done; SnET2 = tin ethyl etiopurpurin.

phase exponential [ $C_t = Ae^{-\alpha t} + Be^{-\beta t}$ ] curves to determine the best fit and evaluate the appropriate pharmacokinetic parameters. In these equations,  $C_t$  is the concentration of compound at a given time;  $A$  and  $B$  are concentration constants;  $\alpha$  and  $\beta$  are distribution and elimination rate constants. The homogeneity and heterogeneity of the photosensitizer within each tissue sample were measured by dividing the sample into five arbitrary extractions and taking three aliquots of each extraction with three repeated emission scans per aliquot. Intrasample and intersample concentration variabilities of the photosensitizers were assessed by drug, tissue, location, and aliquot. A statistical method was applied to the data to identify the major source of variability of photosensitizer concentration by location within a tissue, between types of tissues, and by drug. The first two sources of variability reflect either an undersampling of a population of tumors or variability within the biologic system of a single tissue. The variability of the photosensitizer concentration was assessed by an unpaired Student's  $t$  test for differences in sample extraction and sample for given tissue. The level of significance for this data analysis was set at  $\alpha = .05$ . The photosensitizer concentration data from tumors and adjacent normal tissue were analyzed using two-phase exponential function to deduce the relevant pharmacokinetic parameters. The area under the curve (AUC) for concentration versus time was calculated from time zero to infinity by the trapezoid method. Tissue clearance ( $C_L$ ) was

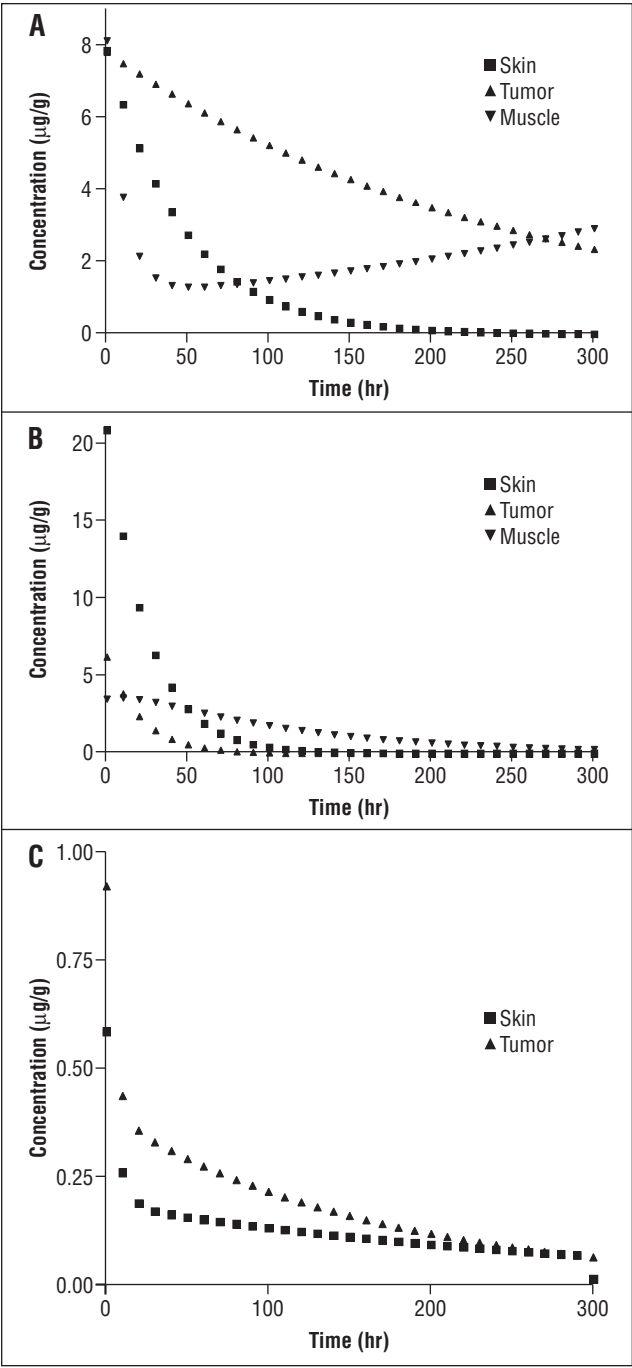
estimated as the dose divided by AUC. The terminal phase elimination half-life  $t_{1/2\beta}$  was given as  $\ln 2/\beta$ .

The likelihood of selective uptake or preferential photosensitizer retention in tumor tissue is the foundation for this study. For these factors to be clinically relevant, there must be a correlation between the tumor and surrounding normal tissue. This was achieved by using the specific uptake ratio (SUR),<sup>16</sup> defined as

**TABLE 3. Pharmacokinetic Parameters for Photosensitizers in Different Tissues of Dogs**

Parameters	Skin	Tumor	Muscle
<b>Porfimer Sodium</b>			
$C_L$ (L/kg/hr)	0.021	0.003	0.011
AUC ( $\mu\text{g/g/hr}$ )	142.050	1075.000	279.100
$t_{1/2\beta}$ (hr)	32.230	175.300	7.210
A ( $\mu\text{g/g}$ )	2.920	3.560	1.060
$\alpha$ (/hr)	0.021	0.004	0.003
B ( $\mu\text{g/g}$ )	4.950	4.260	7.090
$\beta$ (/hr)	0.021	0.004	0.096
<b>SnET2</b>			
$C_L$ (L/kg/hr)	0.001	0.006	0.009
AUC ( $\mu\text{g/g/hr}$ )	644.800	132.700	86.710
$t_{1/2\beta}$ (hr)	17.590	14.460	31.250
A ( $\mu\text{g/g}$ )	9.570	3.000	0.900
$\alpha$ (/hr)	0.040	0.048	0.024
B ( $\mu\text{g/g}$ )	11.350	3.240	0.870
$\beta$ (/hr)	0.039	0.048	0.022
<b>AICIPc</b>			
$C_L$ (L/kg/hr)	0.003	0.004	ND
AUC ( $\mu\text{g/g/hr}$ )	78.090	56.260	ND
$t_{1/2\beta}$ (hr)	4.310	3.340	ND
A ( $\mu\text{g/g}$ )	0.180	0.390	ND
$\alpha$ (/hr)	0.003	0.006	ND
B ( $\mu\text{g/g}$ )	0.400	0.530	ND
$\beta$ (/hr)	0.160	0.207	ND

A and B = concentration constants;  $\alpha$  and  $\beta$  = first rate constants; AICIPc = aluminum chlorophthalocyanine; AUC = area under the concentration versus time curve; CL = tissue clearance; ND = not done; SnET2 = tin ethyl etiopurpurin;  $t_{1/2\beta}$  = elimination half-life.



the ratio of the extracted concentration ( $\mu\text{g/g}$ ) and the injected dose ( $\text{mg/kg}$ ) to investigate the selectivity of the photosensitizers. The SUR is independent of the injected dose but dependent on the time after injection. All analyses were performed with GraphPad Prism (GraphPad Software), and Prophet statistical software (BBN Technology, NIH).

**RESULTS**

The concentration extraction of photosensitizer per sample was not significant. Multiple extractions per sample did not contribute to the overall variability. Intersample and interanimal variability from each photosensitizer was significant ( $P < .05$ ), however. Concentrations of the three photosensitizers in dogs following IV administration are shown in Figure 1. Each point represents the average of at least three extractions from each of three aliquots per tissue sample. The concentration time curve was very similar for the three photosensitizers. The corresponding double exponential non-linear regression coefficients ( $R^2$ ) for each photosensitizer in fibrosarcoma and normal tissue are listed in Table 2. There was only minor improvement in  $R^2$  determined by the double-exponential computa-

**Figure 1.** Time versus porfimer sodium concentration in tumor and normal tissues of dogs treated with porfimer sodium (3 mg/kg IV) (A), tin ethyl etiopurpurin (0.83 mg/kg IV) (B), or aluminum chlorophthalocyanine (0.209 mg/kg IV) (C).

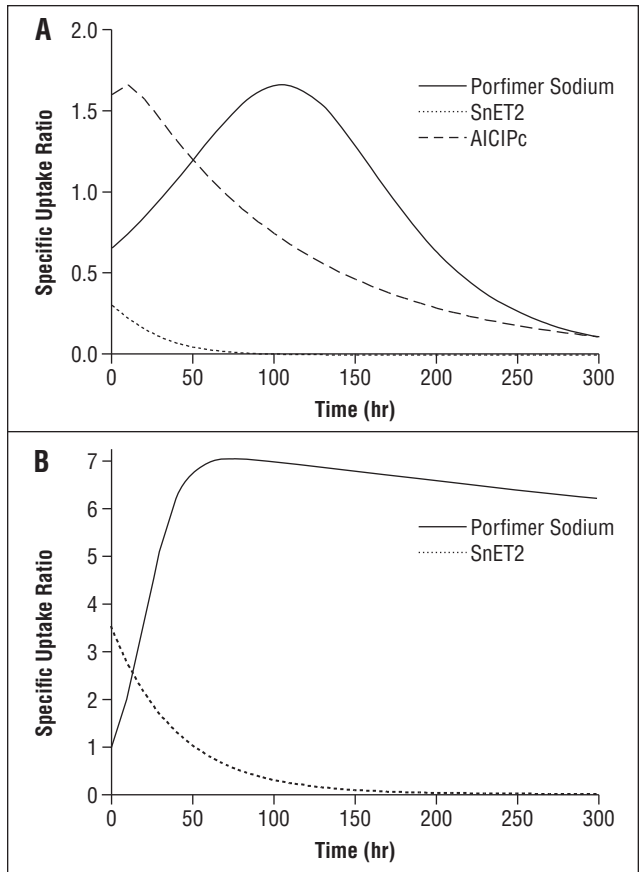
tion. A comparative summary of the pharmacokinetic parameters of each photosensitizer in fibrosarcoma and normal tissue is shown in Table 3. The half-life of porfimer sodium in fibrosarcoma was approximately 12 and 50 times longer than that of SnET2 and AlCIPc, respectively. A similar observation was made for porfimer sodium in skin samples (two and eight times longer than in SnET2 and AlCIPc, respectively). In addition, porfimer sodium had the highest AUC in tumors of the three photosensitizers and among all samples tested. The selectivity of different photosensitizers for tumors located in normal tissue, using SUR as a function of time, is illustrated in Figure 2.

## DISCUSSION

Most preclinical studies of photosensitizers have employed transplantable rodent models. There have been some concerns that rodent tumors are highly inbred systems and are overly homogeneous. Spontaneous tumors in pets resemble human tumors in terms of biologic behavior, vascularity, natural history, and cells, kinetics, clinical stages, histology type, and grade.

Although the exponential phase did not improve the fitting of the data because of poor nonlinear correlation, the concentration versus time curves show clearly that slow distribution is followed by prolonged elimination. In addition, intrasample variability was small, and hence, the extraction procedure was reproducible. However, intersample and interanimal variability were the primary sources of biologi-

cal heterogeneity. All tumor samples tested had the same histology. Nevertheless, they were different in terms of SUR, illustrating the heterogeneity of photosensitizer uptake from tumor to tumor having similar histology. In addition, comparison among tumor samples or normal tissue samples for the same photosensitizer revealed significant ( $P < .05$ ) SUR variability. The source of the variability in normal tissue might arise from macroscopic variability in the cellular components of the section of muscle sampled. As much as possible, tissues that were



**Figure 2.** Time versus tumor:skin ratio for porfimer sodium, tin ethyl etiopurpurin (SnET2), and aluminum chlorophthalocyanine (AlCIPc) (A) and time versus tumor:muscle ratio for porfimer sodium and SnET2 (B).

harvested were uniform. However, it is possible that some samples contained more vascular cells and others contained more muscle tissue. The SUR variation is independent of dose, but may result from the location of these normal tissue samples near or adjacent to tumor sites. This variability in SUR as well as differences in the time after injection that the tissue was harvested may have influenced the photosensitizer uptake. It is possible that a host immune reaction involving vascular cells would permit more photosensitizers to accumulate. Unfortunately, the samples were consumed in assay procedures, so this theory could not be tested. However, it is known that the photosensitizer uptake increases in cells of vascular origin and significant inflammatory response to tumor in an experimental model.<sup>17</sup> In either event, since these were spontaneous tumors in dogs with a normal immune system, the same variability would be expected in human patients. Therefore, the method of in situ measurements would be helpful in reducing toxicity in the human trial.

An important application of this study was to correlate the pharmacokinetic parameters between tissue types for the different photosensitizers as well as for the same photosensitizer in samples of the same type of tissue. The combination of slow tumor and fast normal tissue elimination would provide maximum photosensitizer selectivity with a higher AUC. The route of elimination for these photosensitizers is mainly through feces.<sup>18</sup> Elimination half-life values calculated from the double exponential phase were comparable to other published reports. The half-life of porfimer sodium in canine tumor tissue (175.3 hr) was similar to those previously published (4 to 220 hr)<sup>19</sup> and was 2.5-fold lower than that calculated from a human tumor<sup>20</sup> and six times higher than that for tumors in mice.<sup>21</sup> This stability may be due to the slow process of porfimer

sodium redistribution from tissue, which accounts for the longer half-life and larger AUC. SnET2 elimination half-life (14.46 hr) in tumor tissue is in agreement with previously published data (5.1 to 16.4 hr)<sup>5</sup> and slightly longer than that (11.44 hr) in canine serum.<sup>22</sup> AIClPc is characterized by a relatively short half-life in either tumor (3.34 hr) or skin tissue (4.31 hr). This indicates that AIClPc is eliminated faster than SnET2 from tumor tissue. The half-life of AIClPc determined in the present study is consistent with the value 2.6 hours determined in previous work<sup>6</sup> and is consistent with the half-life of other phthalocyanine derivatives (6.8 to 8.5 hr).<sup>23,24</sup> The elimination half-life of AIClPc (4.32 hr) in skin samples was lower than that for porfimer sodium (32.23 hr) or SnET2 (17.59 hr). This pattern should minimize the risk of skin photosensitization because AIClPc absorbs ambient light much less efficiently than porfimer sodium.<sup>25</sup> Photosensitizer localization and clearance depend on the tissue ability to metabolize and eliminate the photosensitizer. Clearance was slower for porfimer sodium and SnET2 in tumor tissue compared with clearance from normal tissue. The slow clearance of these compounds from tumor tissue may depend on the low accessibility of photosensitizer binding sites to the protein carriers and to the poor lymphatic drainage of tumor tissue.<sup>26</sup>

Considering both tissue types and all photosensitizers tested, porfimer sodium had the highest AUC. This finding supported the affinity of porfimer sodium for tumor tissue. It is believed that a subpopulation of tumor-associated macrophages accumulate a higher level of porfimer sodium.<sup>27</sup> The value determined in the present study was 2.5 times lower than that reported in human tumors.<sup>20</sup> The ratio of tumor:normal tissue was determined to be in the range of 4 to 10, suggesting a potential selectivity for PDT treatment of tumors

located in these normal tissues. However, the intrinsic sensitivity of tumor and normal tissues towards porfimer sodium-mediated PDT needs to be quantified and considered for evaluations of PDT effectiveness. The skin sample from SnET2 treatment had the highest AUC of all skin samples tested. The ratio of tumor:skin was 0.02, indicating a poor selectivity. AICIPc had the lowest AUC of the three photosensitizers evaluated, reflecting its lower uptake in both tumor and normal tissue. The variation in pharmacokinetic values obtained in the present study versus those found by other investigators was likely due to the use of a biopsy sample versus actual living large animal where serum was collected and evaluated at different times. The data analyzed in this study were from tissue homogenate, a mixture of intracellular, extracellular, lymphatic, and vascular tissue. Studies in animal models have demonstrated serum concentrations are a better predictor than tissue homogenate levels because of photosensitizer homogeneity.<sup>8,10,13</sup>

The degree of selectivity for a photosensitizer is usually expressed by the ratio of its concentration in the tumor to that in select normal tissues. The tumor:normal tissue ratio is essential in predicting the risk of damage to the normal tissue surrounding a tumor during PDT. For tumors located on skin tissue the SUR of porfimer sodium was less than one between 35 and 175 hours and reaches a peak at 100 hours. This should be the most favorable time for PDT treatment, where multiple treatments can be performed following a single IV injection of porfimer sodium. The SUR for AICIPc was less than one for times less than 75 hours and reaches an early peak at 11.5 hours, which appeared to be the ideal treatment time for that compound. SnET2 may not be a good choice for treatment of tumors located in the skin due to the pharmacokinetic results and the SUR (less than one at all times). For tu-

mors located on muscle tissue, both porfimer sodium and SnET2 have acceptable treatment parameters. In muscle tissue, the SUR for SnET2 was greater than one for times less than 50 hours. Therefore, the ideal treatment time should be very soon after administration. Porfimer sodium remains the ideal photosensitizer due to the SUR greater than one over a long period of time, which could allow repeated phototreatments.

## CONCLUSION

The common problem of obtaining matched normal and tumor tissue biopsies from human subjects for photosensitizer pharmacokinetics studies was overcome in this study by using dogs with spontaneous tumors. Both nonlinear regression curves were used to fit the photosensitizer concentration data, which resulted in poor correlation. Nevertheless, the double exponential phase model could characterize both distribution and elimination processes. Neither model performed well in tumors with porfimer sodium and AICIPc, mostly due to the very large intersample and interanimal variability. Variability in photosensitizer SUR was present between tissues and among animals within the same photosensitizer. Light doses can be individually tailored at the time of therapy to compensate for this randomness.

The study showed that porfimer sodium presented an improved selectivity and could be more effective for tumors located either in the skin or in the muscle. However, one cannot conclude that porfimer sodium is necessarily a better treatment than SnET2 or AICIPc, despite its good selectivity, because SnET2 and AICIPc are activated at 662 and 675 nm, respectively, which provides better light transmission in tissue than porfimer sodium. This is an attempt to present a real case scenario and provide useful information for the clinician for a better photosensitizer choice and therapeutic

window for tumors located in different sites of normal tissue. At present, further studies are underway to model the data and provide accurate predictions on the photosensitizer SUR in specific tissue before PDT treatment.

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