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A safety study of a novel photosensitizer, sinoporphyrin sodium, for photodynamic therapy in Beagle dogs†

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Sinoporphyrin sodium (DVDMS) is a novel hematoporphyrin-like photosensitizer developed for photodynamic therapy (PDT), an effective therapeutic modality for tumor treatment; however, the safety of photosensitizer-based PDT is always of great concern. The purpose of the current study was to investigate the potential repeated-dose toxicity and describe the toxicokinetic process of DVDMS-based PDT in Beagle dogs. The dogs were randomly allocated to six groups, and then were administrated a DVDMS preparation intravenously at dose levels of 0, 1, 3, 9, 1 and 9 mg per kg body weight, respectively; then, the latter two groups were illuminated 24 h later with a 630 nm laser for 10 min, once every seven days for 5 weeks. During the study period, clinical signs, mortality, body weight, food consumption, body temperature, ophthalmoscopy, hematology, serum biochemistry, urinalysis, electrocardiograms, toxicokinetics, organ weights, gross anatomy and histopathology were examined. After the administration, no deaths were observed; however, the dogs that received PDT showed skin swelling and ulceration, indicating that DVDMS-PDT induced a phototoxic effect. DVDMS led to an increase in blood coagulation in dogs in the 9 mg kg^{-1} group and in the two PDT groups on Day 35, whereas it induced a decrease in dogs in the 3 mg kg^{-1} group and in the two PDT groups on Day 49. The toxicokinetic study showed that the systematic exposure of DVDMS in dogs occurred in a dose-dependent manner, and DVDMS did not accumulate in blood plasma. The DVDMS-based PDT group showed no obvious treatment-related pathological changes; however, slight or mild brown-and-yellow pigmentation of DVDMS (or its metabolite) was observed to deposit in the liver, spleen, local lymph nodes and marrow of dogs in the mid- and high-dose groups, as well as the high-dose PDT group. In females, the absolute and relative spleen weights increased in dogs in the 9 mg kg^{-1} DVDMS groups with and without PDT during the treatment and recovery period, respectively. The target organs are presumed to be the liver and immune organs (spleen, bone marrow and lymph nodes), while all of the responses were slight. Based on the results above, the no-observed-adverse-effect level (NOAEL) was considered to be 1 mg kg^{-1}, and DVDMS-PDT appeared to be a safe and promising anti-tumor therapy in the clinic.

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1. Introduction

Photodynamic therapy (PDT) is a well-established therapeutic modality for the treatment of tumors. PDT has been clinically applied to a variety of tumors, such as lung, cervical, ovarian,[1] early esophageal,[2] head and neck,[3] bladder[4] and melanoma[5] cancers. The effect of PDT on cancer is based on the preferential uptake of photosensitizers in tumor cells or tissues, and subsequent activation of the photosensitizers by an appropriate wavelength of laser light, which leads to a generation of reactive oxygen species (ROS), resulting in the killing of tumor cells by inducing cellular apoptosis, necrosis,[6] autophagy,[7] immune inflammatory responses[8] and damage to the microvasculature[9] of tumors. The greatest benefit of PDT is its high tumor-targeting, which is due to the preferential accumulation of photosensitizers in tumor cells and the selective irradiation with a specific wavelength of laser light.[10] PDT is considered to be a safe, well-tolerated and effective treatment for cancer; moreover, it also has some limitations, such as the limited penetration of light into deep tumor tissues.[11]

The photosensitizer is a key factor in PDT. Hematoporphyrin derivative (HpD), a complex mixture of porphyrins

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derived from hematoporphyrin, has been used for localization and photodynamic therapy of tumors;[12] furthermore, various forms of HpD have been commercially available for over thirty years, and it has been used to treat thousands upon thousands of patients.[13] Recently, more new derivatives of porphyrin have become available for the treatment of cancer;[14] for instance, Photofrin®, the commercially available photosensitizer derived from HpD, which has been approved by the FDA as a photosensitizer in PDT cancer therapy, is currently the most widely used photosensitizer.[15] However, its side-effects should not be ignored. Photofrin® is a mixture of dimers and oligomers of hematoporphyrin, in which the porphyrin units are linked by ether, ester and C–C bonds. The first drawback is that the esters of ethers were found to be unstable at room temperature, and hydrolysis was also detected in the injection solution within a few minutes, which limited its clinical applications.[16] Photofrin®, composed of multiple components, has no controllable quality standards.[17] Moreover, due to its strong toxicity to skin and eyes, patients are warned to avoid exposure of skin and eyes to direct sunlight or bright light indoors for 4–6 weeks following injection with Photofrin®.[18] To overcome the limitations of Photofrin®, Fang and Yang at the Chinese Academy of Medical Sciences isolated an active compound from Photofrin II named sinoporphyrin sodium, also known as DVDMS. DVDMS is patented in the People's Republic of China.[19] DVDMS, a newly developed, novel photosensitizer with a single and well-defined chemical structure, has shown a much higher photo-activity than the commonly used clinical photosensitizer Photofrin® in preclinical studies.[20] In our previous study on the effect of DVDMS-PDT on the mouse S180 sarcoma model, the tumor weight inhibition rates treated under irradiation conditions (38, 76, 152 J cm^{-2}) 24 h after DVDMS (2 mg kg^{-1}) injection were 82.83%, 88.56%, and 95.59% ($P < 0.05$), respectively.[21] One study has reported that DVDMS-PDT showed a cell proliferation inhibition effect on Eca-109 cells, which are a human esophageal cancer cell line. Furthermore, when Eca-109 cells were treated with DVDMS (5 μg ml^{-1}) and irradiated, the maximal uptake of DVDMS occurred within 3 hours *in vitro*, with a mitochondrial sub-cellular localization.[22] DVDMS was observed to have higher fluorescence intensity and singlet oxygen production efficiency when compared with other porphyrin-like photosensitizers.[23] In addition, DVDMS was proved to have good targeting ability for tumor cells or tissues.[24,25] Additionally, DVDMS has greater water-solubility and stability, and has a single chemical structure.

The research above indicated that DVDMS-PDT is a promising therapeutic modality for tumor treatment. However, there are some safety risks regarding the clinical application of the existing hematoporphyrin-like photosensitizers. Hp and HpD, for example, may cause prolonged photosensitivity and toxicity in normal tissues, due to their long clearance time.[26] According to an earlier study, animal trials showed that DVDMS preferentially accumulated in tumor tissues, was metabolized quickly in normal tissues and displayed slight skin phototoxicity in mice.[21,25] However, the literature pertaining to the *in vivo* toxicity and toxicokinetics of DVDMS is absent. For clinical purposes, a safety evaluation of DVDMS has been conducted in repeated-dose toxicity and toxicokinetic studies of DVDMS in Beagle dogs through intravenous administration. This study was performed under Good Laboratory Practice regulations.

2. Materials and methods

2.1 Test chemicals and reagents

Sinoporphyrin sodium (DVDMS) (Fig. 1), Lot: 20121019, was supplied as a lyophilized powder by Qinglong High Technology Co. Ltd. It was a dark red-violet crystalline powder. The chemical identity of DVDMS was confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy, infrared (IR) spectroscopy and mass spectroscopy (MS). A purity of >98.68% was determined by gas chromatography (GC) and GC-MS. The chemical was stored in the dark at 0–10 °C. DVDMS lyophilized powder was prepared in dark and sterile conditions, and was dissolved immediately in a 0.9% saline solution before use. The prepared solution was stored in the dark and used as soon as possible. The 0.9% normal saline, manufactured by Shandong Hualu Pharmaceutical Co. Ltd, Lot: 120330703, was set as a negative control.

2.2 Animals and husbandry

Forty-eight male (weighing 6.2–8.0 kg) and female (weighing 6.0–8.5 kg) Beagle dogs (conventional degree, Certificate No. SCXK (Jing) 2007–0003), aged 6–12 months and healthy, sexually-mature, and unmated, were purchased from Beijing KeYu Animal Husbandry Center. They were clinically examined for general health conditions and parasite infestation to confirm their suitability for the study, and allowed to acclimate to the laboratory environment for 26 days prior to treatment. Open conditions were maintained in animal room, with a 12 h light–dark cycle at 19.2–25.1 °C temperature and 42%–59% relative humidity. The dogs were housed individually in stainless steel cages and allowed free access to solid chow and tap water. Food was restricted to 300 g per day, and any remaining, unconsumed food was weighed to calculate the net food intake per animal. This experiment was performed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC Inter-

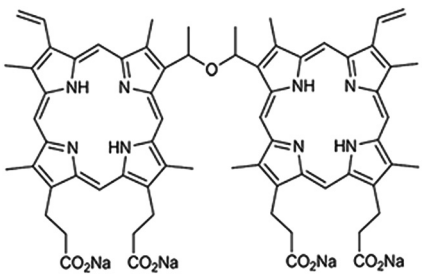

Fig. 1 Chemical structure of DVDMS.

national), and the animals were maintained in accordance with the 'Guide for the Care and Use of Laboratory Animals'.

2.3 Study design and dose selection

After the quarantine-acclimation period, four dogs per sex were allocated to one of the six different dose groups by computerized random selection to achieve similar group mean body weights. The six dose groups were as follows: 0 (0.9% saline control), 1 mg kg^{-1} DVDMS, 3 mg kg^{-1} DVDMS, and 9 mg kg^{-1} DVDMS, as well as 1 mg kg^{-1} DVDMS-based PDT and 9 mg kg^{-1} DVDMS-based PDT (Table 1). DVDMS preparation or 0.9% saline was injected *via* the forelimb vein at an infusion rate of less than or equal to 2 ml min^{-1} once a week, 5 successive times, without anesthesia. The dosing volume was 5 ml kg^{-1} in all groups. The animals in the two PDT groups were fixed, and then the fur surrounding the injection sites were shaved off in an area of about 16 square centimeters, exposing the skin around the injection sites before insertion of the needle. Twenty-four hours after intravenous administration of DVDMS, we shaved off the fur on the backs of the dogs in an area of about 16–25 square centimeters to fully expose the irradiated sites. Then, the backs of the dogs in the two PDT groups were photo-irradiated for 10 minutes using a PDT 630 II Type Laser Photodynamic Therapy Instrument emitting red light at 630 nm by a lensed fiber. The energy density at the illumination area was 76 J cm^{-2}, and the power of the laser was 100 mW. The spot diameter was 1 cm and the illumination area was 0.785 cm^2. The dogs were housed in the animal room with subdued lighting after administration of DVDMS. Four animals per group, half of the males and females, were anesthetized with an intravenous injection of 2% pentobarbital and were killed for pathological examination at the end of the treatment period on Day 35, and the remaining half of the dogs were killed and examined at the end of the two week recovery period on Day 49.

The selection of the dose levels employed in the present study was based on data regarding anticipated human clinical application and the results of an experimental study on the anticancer effect of DVDMS-PDT on the mouse S180 sarcoma model. In a clinical therapeutic plan, DVDMS is intended to be administered through intravenous infusion at a dose of 0.2 mg kg^{-1}, with a one-time treatment in general and additional administration if necessary. In the present study, 9 mg kg^{-1} was specified as the highest dose level, and the lower doses were 3 and 1 mg kg^{-1}, which were, respectively, 2.8, 8.3 and 25 times the clinical application dosage, using a common ratio of 3.

2.4 Clinical observations

Throughout the time period, all the animals were observed for clinical signs of toxicity, morbidity and mortality once or twice daily, before and after administration of irradiation. During the two week convalescence period, the remaining animals were observed for reversibility, persistence, and delayed occurrence of toxic effects. Daily cage side observation during predose and post-dose periods included appearance, fur, activity condition, neural responses, respiratory status, and posture. Detailed clinical examinations were made outside the cage to check for abnormalities of the neck, head (involving eyes, ears, mouth and nose), hypogastrium, anus, perineum, color of skin and muscle tone, in addition to trauma and tumor. The injection site was examined after administration, involving the responses of erythema, dropsy, scleroma, ulceration and pyorrhea.

2.5 Body weight, food consumption and body temperature changes

The body weights and temperatures of all animals were measured twice during the quarantine period, and once every week during the administration and recovery periods. Food consumption was measured twice during the quarantine period and at weekly intervals during treatment and convalescence. The amounts of food (approximately 300 g) were recorded before they were supplied to each cage, and the remaining food was measured the next day to determine the differences, which were regarded as daily food consumption (g per 100 g bodyweight).

2.6 Laboratory investigation

Blood and urine samples were collected from all dogs twice before administration and weekly during the treatment and recovery periods. All the animals were fasted overnight prior to obtaining blood or urine samples. Then, the blood samples were drawn from forelimb vena into evacuated blood collection tubes, and urine was collected from the bladder onto a specimen test paper through a catheter. EDTA and sodium citrate

Table 1 The dosage regimen design of Beagle dogs injected intravenously with DVDMS for 30 days[a]

Group	Dose level (mg kg^{-1})	Energy density (J cm^{-2})	Multiple of equivalent to clinical dose	Animal number ♀	Animal number ♂
I Control group	0	—	0	4	4
II Low-dose group	1	—	2.8	4	4
III Middle-dose group	3	—	8.3	4	4
IV High-dose group	9	—	25	4	4
V Low-dose-PDT group	1	76	2.8	4	4
VI High-dose-PDT group	9	76	25	4	4

[a] The clinical quasi dosage in humans is 0.2 mg kg^{-1}. The dosage conversion coefficient according to body weight from human to dog is 1.8.

were used as anticoagulants for blood coagulation examination.

2.6.1 Hematology. Hematological estimations were carried out using a MEK-7222 K Automated Hematology Analyzer (Nihon-Kodhen Co., Tokyo, Japan). The test parameters included leukocyte count (WBC), erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width-variation coefficient (RDW-CV), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), neutrophilic granulocyte (NEUT), lymphocyte (LYM), monocyte (MON), eosinophil (EOS), basophilic granulocyte (BAS), reticulocyte (Reti). Blood smears were stained with Wright-Giemsa and brilliant-cresyl-blue,[27] and the Reti count was counted under an Olympus microscope.

2.6.2 Serum biochemistry and electrolytes. Serum biochemistry and electrolyte examinations were conducted with a Beckman Coulter Autoanalyzer (Beckman Coulter International Inc., U.S.A.) and an AVL-9181 Electrolyte Analyzer (AVL Scientific Co., Roswell, Georgia, U.S.A.). The parameters measured were: alanine aminotransferase/glutamic pyruvic transaminase (ALT/GPT), aspartate aminotransferase/glutamic oxaloacetic transaminase (AST/GOT), total protein (TP), albumin (ALB), globulin (GLB), albumin-globulin ratio (A/G), total bilirubin (TBIL), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK), blood glucose (GLU), urea (UREA), uric acid (UA), creatinine (CRE), cholesterol (CHO), triglyceride (TG), calcium (Ca), potassium (K), sodium (Na), and chlorine (Cl).

2.6.3 Blood coagulation. The parameters of blood coagulation, including thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (Fbg), were calculated using a DIAGNOSTICA STAGO STA-4 Coagmaster (Junior Instruments Co., Gennevilliers, France).

2.6.4 Electrocardiography. The II-lead electrocardiography was recorded using an ECG-9130P 12-lead Electrocardiography Automatic Analysis System (Nihon-Kodhen Co., Tokyo, Japan), and the parameters of heart rate, PR duration, QT duration and QRS duration were calculated. The electrocardiography was recorded at the end of the treatment period (Day 35) and after the convalescence period (Day 49).

2.6.5 Urinalysis. Urinalysis was performed using Multistrix® Strips (Bayer Corp., Bridgend, South Wales, UK), and the results were recorded using a Combi Scan 500 Urine Analyzer (Analyticon Biotechnologies AG, Lichtenfels, Germany). The urinalysis parameters measured were specific gravity (SG), leukocyte (LEU), nitrite (NIT), pH, erythrocyte (ERY), protein (PRO), glucose (GLU), ascorbic (ASC), ketone (KET), urobilinogen (UBG), and bilirubin (BIL).

2.6.6 Ophthalmoscopy. Ophthalmoscopy examinations on all dogs were carried out immediately before starting the treatment, at the termination of administration (Day 35) and after the convalescence period (Day 49). The observation areas included the corneas, conjunctiva, scleras, irises, lenses, pupils, lids and fundus.

2.7 Toxicokinetics

Blood samples (approximately 1 mL) were collected in heparinized tubes on the day of the first and final dose, respectively. The plasma was separated by centrifugation at 3000 rpm for 15 min and immediately stored at −80 °C. The predetermined time points of blood sampling were: pre-dose and 2 h post-treatment for the control group; pre-dose and 5 min, 30 min, 2 h, 8 h, 24 h, 48 h, 72 h and 96 h after administration for the 5 treatment groups. 50 μl of the plasma samples were spiked with internal standard solution F18 (Institute of Materia Medica, Peking Union Medical College, China; Lot: C2430-022-A2) and deproteinized by adding acetonitrile. The samples were vortex mixed and centrifuged to extract the analyte. Then, 40 μl of supernatant was injected into the HPLC system. Toxicokinetic analysis was performed using Phoenix™ WinNonlin Professional Edition (Pharsight Corporation, Version 5.2.1), and the toxicokinetic parameters were calculated according to the non-compartment model.

2.8 Necropsy and histopathological examination

After the endpoint of the treatment and after the recovery period, 2 animals per group, half of the males and half of the females, were killed under anesthesia conditions with 2% pentobarbital sodium. A through necropsy was performed, and the following organs were removed: brain, heart, liver, kidneys, adrenal glands, thymus, spleen, testes, epididymides, ovaries, uterus and lungs. After dissection to separate the fat and connective tissue, the absolute and relative weights (weight per 100 g body weight) of each organ above were calculated and recorded. Samples of these organs and the spinal cord, hypophysis, thyroid, parathyroid glands, esophagus, stomach, small and large intestine, pancreas, trachea, aorta, breasts, ischiadic nerve, bladder, marrow (sternum), lymph nodes, optic nerve and prostate were fixed in 10% buffered formalin solution. These specimens were embedded in paraffin, sectioned at 3–5 μm, and stained with hematoxylin-eosin for microscopic examination. All gross lesions as defined by the pathologist were also included in the examination.

2.9 Statistical analysis

Quantitative continuous data, such as body weight, food consumption, hematological serum biochemistry parameters, organ weights and urine pH were analyzed by the Kolmogorov-Smirnov test to test normality and the Levene's median test for evaluating homogeneity of variance, and the one-way analysis of variance (ANOVA) was used for the continuous data. If the test of normality and variance homogeneity failed, the Kruskal–Wallis test was employed. If the results of variance analysis showed a significant difference ($P < 0.05$ or 0.01), the Dunnett's Multiple Comparison test was used for all pair-wise comparisons to the control group to identify the statistical significances of individual groups. The statistics were considered to be complete in the case of no statistical significance ($P \geq 0.05$). If the Kruskal–Wallis test was statistically significant, the Mann–Whitney test was employed for pair-wise comparisons,

whereas the statistical analysis was complete. Qualitative data such as urinalysis parameters were statistically evaluated by the Kruskal–Wallis test. If a significant difference emerged ($P <$ 0.05 or 0.01), then the data were compared and analyzed using the Mann–Whitney test to determine which treated groups differed from the control group.

Toxicokinetic analysis of the plasma concentration was performed using Phoenix™ WinNonlin Professional Edition (Pharsight Corporation, Version 5.2.1), and the values of $T_{1/2}$, C_{max} and AUC_{0-t} were calculated according to the non-compartment model. The calculations of average, standard deviation, variance coefficient, C_{max} ratio, AUC_{0-t} ratio and accumulation coefficient were performed in Microsoft Excel (Version 2007).

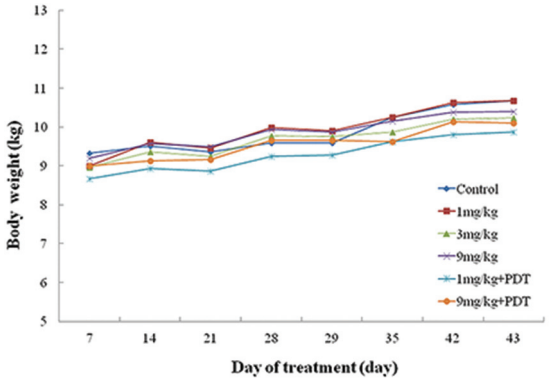

Fig. 2 Body weight of Beagle dogs during the treatment period and recovery period. Significantly different from control group, (※) $P < 0.05$ or (Δ) $P < 0.01$.

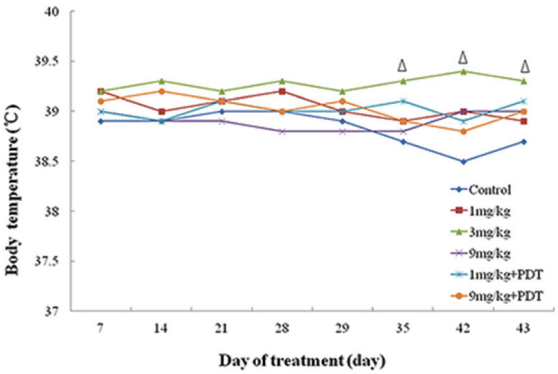

Fig. 3 Body temperature of Beagle dogs during the treatment period and recovery period. Significantly different from control group, (※) $P < 0.05$ or (Δ) $P < 0.01$.

3. Results

3.1 Clinical observations

No deaths were observed cage-side in any group during the administration and recovery periods. The irradiated sites of the dogs, which were treated with 1 mg kg^{-1} DVDMS-based PDT and 9 mg kg^{-1} DVDMS-based PDT showed signs of skin swelling and ulceration. The ulceration and escharotic of skin had not fully recovered at the endpoint of the study. Redness of the conjunctiva was observed in several animals of each treatment group after administration. At the end of the treatment period (Day 35), a veterinarian conducted an ophthalmic examination, and confirmed that only the animals in the 9 mg kg^{-1} DVDMS-based PDT group had conjunctival redness. Moreover, the dogs from other treatment groups, which developed the same symptoms of conjunctival redness returned to normal during the restoration stage. No other abnormal clinical signs were observed cage-side.

3.2 Body weight, food consumption and body temperature changes

There existed no significantly toxicological variations in body weights between the control and treatment groups (Fig. 2). The food consumptions of all five treatment groups did not indicate differences from the control value in either sex throughout the study period. The temperature values of the dogs of the 3 mg kg^{-1} DVDMS group were significantly higher than those of the control group on Day 35, Day 42 and Day 43 (Fig. 3).

3.3 Laboratory investigation

3.3.1 Hematology. A dramatic increase existed in the MCV value of the 3 mg kg^{-1} DVDMS group and in the RDW value of the 1 mg kg^{-1} DVDMS-based PDT group at the end of the administration stage (Day 35). Other indicators revealed no significant abnormality during the treatment period. There was no statistically significant change in any indicators of any groups during the recovery period (Table 2).

3.3.2 Serum biochemistry and electrolytes. After the last administration and irradiation (Day 35), the values of ALB and A/G of the 9 mg kg^{-1} DVDMS group were significantly lower than that of the control group, while the TP and GLB of the 9 mg kg^{-1} DVDMS group were statistically significantly higher than those of the control group. Moreover, a significant decrease in the AST and ALB values in the 1 mg kg^{-1} DVDMS-based PDT group, and of the ALB, A/G, CK, and UREA values in the 9 mg kg^{-1} DVDMS-based PDT group, were observed when compared with the saline control group (Table 3).

On Day 49, the value of Ca in the 3 mg kg^{-1} DVDMS group was significantly lower than that in control group, and the TBIL value of the 9 mg kg^{-1} DVDMS group was dramatically higher than that of the control group at the end of the recovery period. Moreover, the TBIL and CREA values apparently decreased in both the 1 mg kg^{-1} DVDMS-based PDT and 9 mg kg^{-1} DVDMS-based PDT groups when compared with those of the saline control group (Table 3).

3.3.3 Blood coagulation. At the end of the last dosing and irradiation (Day 35), the coagulation tests revealed that the TT values of the 9 mg kg^{-1} DVDMS group, the PT values of the 1 mg kg^{-1} DVDMS-based PDT group, and both the PT and

Table 2 Hematology parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$)[a]

		Dose (mg kg^{-1})					
Date	Parameters	0	1	3	9	1 + PDT	9 + PDT
D35 n = 8	WBC(10^9 L^{-1})	14.41 ± 2.48	15.3 ± 5.6	16.85 ± 3.11	17.86 ± 3.5	15.2 ± 3.44	19.7 ± 8.66
	RBC(10^{12} L^{-1})	6.72 ± 0.27	6.31 ± 0.61	6.31 ± 0.84	6.2 ± 0.39	6.12 ± 0.56	6.16 ± 0.68
	HGB(g L^{-1})	154.12 ± 6.31	145.87 ± 13.37	149.75 ± 22.89	138.75 ± 10.54	140.75 ± 12.35	142.63 ± 8.83
	HCT(%)	41.55 ± 1.53	39.5 ± 3.81	40.05 ± 5.21	37.41 ± 2.39	37.59 ± 2.81	38.56 ± 2.69
	MCV(fL)	61.9 ± 1.14	62.56 ± 1.63	63.49 ± 0.99*	60.34 ± 2.25	61.49 ± 1.86	62.89 ± 2.82
	MCH(pg)	22.98 ± 0.66	23.14 ± 1.01	23.69 ± 0.64	22.34 ± 0.63	22.99 ± 0.77	23.3 ± 1.24
	MCHC(g L^{-1})	371 ± 7.03	369.38 ± 9.23	373 ± 10.35	370.63 ± 8.77	374.13 ± 7.22	370.13 ± 10.49
	RDW (%)	13.79 ± 0.74	13.83 ± 0.72	13.99 ± 0.82	13.69 ± 0.99	15.25 ± 1.19**	13.34 ± 0.76
	PLT(10^9 L^{-1})	312.75 ± 82.43	254.75 ± 80.1	239.88 ± 87.36	302 ± 99.65	370.88 ± 95.8	361.25 ± 96.13
	PCT(%)	0.28 ± 0.06	0.22 ± 0.08	0.23 ± 0.08	0.27 ± 0.07	0.29 ± 0.1	0.3 ± 0.07
	MPV(fL)	9.21 ± 1.66	8.89 ± 1.12	9.85 ± 1.32	9.16 ± 1.62	7.84 ± 1.09	8.43 ± 1.23
	PDW(%)	16.44 ± 0.73	16.04 ± 1.12	16.11 ± 0.8	16.25 ± 1.49	16.08 ± 0.85	16.7 ± 0.89
	LYM (%)	42.79 ± 8.33	37.22 ± 11.39	35.99 ± 13.5	38.98 ± 7.58	37.87 ± 9.93	40.86 ± 14.7
	MON(%)	0.8 ± 0.32	0.74 ± 0.46	0.91 ± 0.65	0.75 ± 0.26	0.64 ± 0.11	0.83 ± 0.4
	NEUT(%)	56.38 ± 8.38	61.79 ± 11.97	63.06 ± 13.97	60.25 ± 7.64	61.47 ± 9.99	58.3 ± 14.93
	EOS(%)	0.04 ± 0.05	0.25 ± 0.63	0.04 ± 0.05	0.03 ± 0.05	0.01 ± 0.04	0.01 ± 0.04
	BAS(%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Reti($^0/_{00}$)	8.38 ± 2.83	8 ± 1.69	8.25 ± 1.28	7.5 ± 1.6	7.63 ± 2.62	7 ± 1.51
D49 n = 4	WBC(10^9 L^{-1})	16.73 ± 1.65	15.78 ± 4.57	16.52 ± 3.17	17.23 ± 0.89	17.43 ± 1.39	18.28 ± 2.07
	RBC(10^{12} L^{-1})	6.86 ± 0.24	6.66 ± 0.62	6.37 ± 0.46	6.32 ± 0.26	6.54 ± 0.4	6.28 ± 0.72
	HGB(g L^{-1})	166.25 ± 6.7	162.5 ± 17.64	160 ± 14.26	144.25 ± 8.3	156.5 ± 5.92	149.25 ± 11.15
	HCT(%)	43.55 ± 1.29	42.35 ± 4.12	41.38 ± 2.67	38.42 ± 1.78	41.43 ± 1.82	40.03 ± 2.56
	MCV(fL)	63.5 ± 1.37	63.58 ± 1.81	64.98 ± 0.7	60.78 ± 1.14	63.4 ± 2.92	64.03 ± 3.28
	MCH(pg)	24.28 ± 0.67	24.37 ± 0.89	25.1 ± 0.88	22.8 ± 0.62	23.98 ± 1.27	23.85 ± 1.01
	MCHC(g L^{-1})	382 ± 4.76	383.5 ± 4.2	386.25 ± 13.07	375.5 ± 4.65	378 ± 7.53	372.5 ± 4.2
	RDW (%)	13.33 ± 0.65	13.83 ± 0.33	13.45 ± 1	13.45 ± 0.45	14.03 ± 0.99	13.45 ± 0.53
	PLT(10^9 L^{-1})	284 ± 65.26	257 ± 48.81	281.25 ± 47.3	291.5 ± 63.15	286.5 ± 78.53	364 ± 91.55
	PCT(%)	0.26 ± 0.04	0.26 ± 0.05	0.25 ± 0.06	0.3 ± 0.08	0.23 ± 0.06	0.32 ± 0.05
	MPV(fL)	9.28 ± 1.6	9.9 ± 0.37	8.95 ± 2.13	10.33 ± 1.11	8.07 ± 0.62	9.1 ± 1.75
	PDW(%)	17.4 ± 0.75	16.18 ± 0.53	17.98 ± 2.77	16.18 ± 0.45	16.6 ± 0.88	16.9 ± 0.5
	LYM (%)	51.45 ± 8.61	42.9 ± 10.22	44.4 ± 16.27	47.55 ± 4.49	35.2 ± 6.36	48.53 ± 7.89
	MON(%)	0.73 ± 0.46	1.08 ± 0.71	0.8 ± 0.36	0.63 ± 0.3	0.75 ± 0.19	0.93 ± 0.34
	NEUT(%)	47.43 ± 9.03	56 ± 10.9	54.8 ± 16.62	51.8 ± 4.4	64 ± 6.49	50.53 ± 8.2
	EOS(%)	0.4 ± 0.67	0.03 ± 0.05	0 ± 0	0.03 ± 0.05	0.05 ± 0.06	0.03 ± 0.05
	BAS(%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Reti($^0/_{00}$)	10.25 ± 2.75	6.5 ± 1.29	7.5 ± 1.29	8.5 ± 2.08	8 ± 1.41	8.5 ± 1.29

[a] Values are presented as mean ± SD. *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

APTT values of the 9 mg kg^{-1} DVDMS-based PDT group had a tendency to increase and showed a statistical difference from the saline control group (Table 4).

After the end of the convalescence period (Day 49), however, the results indicated that there was a significant decrease in the APTT values in the 3 mg kg^{-1} DVDMS group, the 1 mg kg^{-1} DVDMS-based PDT group and the 9 mg kg^{-1} DVDMS-based PDT group when compared with the APTT values of the control group (Table 4).

3.3.4 Electrocardiography. By the end of administration (Day 35), the QRS duration of the two DVDMS-based PDT groups had a tendency toward decrease and showed a statistical difference with the control group. After the recovery period, the heart rate, PR duration, QRS duration and QT duration of all the animals in each treatment group revealed no abnormality (Table 5).

3.3.5 Urinalysis. In urinalysis, when finishing the treatment (Day 35), the pH value of the 1 mg kg^{-1} DVDMS-based PDT group was obviously lower than that of the saline control group (Table 6).

At the end of convalescence (Day 49), the urine of only one animal in the 3 mg kg^{-1} DVDMS group appeared brown and turbid, and the value for ERY was strongly positive (+++). Moreover, the ERY of the 9 mg kg^{-1} DVDMS-based PDT group was observed to be strongly positive (+++) as well (Table 6).

3.3.6 Ophthalmoscopy. Conducting the ophthalmoscopic examination shortly after the last treatment (Day 35), the animals in the 9 mg kg^{-1} DVDMS-based PDT group appeared to have a slight flush on their palpebral conjunctivas, and some of them were observed to have bulbar conjunctiva congestion. Ophthalmoscopic examination of other treatment groups did not reveal any ocular changes shortly after the last dosing and irradiation, and shortly after convalescence.

3.4 Toxicokinetics

The toxicokinetic parameters are summarized in Table 7.

Table 3 Serum biochemistry and electrolyte parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$)[a]

		Dose (mg kg^{-1})					
Date	Parameters	0	1	3	9	1 + PDT	9 + PDT
D35 n = 8	ALT(U l^{-1})	46.63 ± 12.78	38.5 ± 9.56	44.63 ± 11.49	54.5 ± 28.41	42.62 ± 11.01	42.88 ± 7.79
	AST(U l^{-1})	45.5 ± 3.42	45.63 ± 5.66	46.88 ± 8.08	50.88 ± 10.84	37.13 ± 2.7**	50.63 ± 9.21
	TP(g l^{-1})	71.89 ± 4.8	67.94 ± 1.96	70.3 ± 4.33	76.99 ± 5.06*	70.88 ± 2.28	73.13 ± 4.06
	ALB(g l^{-1})	32.39 ± 1.21	31.71 ± 1.18	32.21 ± 2.55	30.64 ± 1.45*	30.36 ± 2.01*	30.39 ± 1.12*
	GLB(g l^{-1})	39.5 ± 4.42	36.23 ± 1.62	38.09 ± 2.58	46.35 ± 5.08*	40.51 ± 3.91	42.74 ± 3.31
	A/G	0.76 ± 0.09	0.84 ± 0.05	0.8 ± 0.08	0.61 ± 0.1*	0.74 ± 0.12	0.68 ± 0.05*
	TBIL(μmol l^{-1})	3.14 ± 0.41	2.69 ± 0.27	2.8 ± 0.48	2.81 ± 0.32	2.6 ± 0.33	2.98 ± 0.36
	ALP(U l^{-1})	128.25 ± 26.49	119.25 ± 25.22	119.88 ± 37.78	122.5 ± 32.89	112.5 ± 40.86	116.88 ± 20.4
	GGT(U l^{-1})	2.87 ± 0.35	2.87 ± 0.35	3.38 ± 0.92	2.88 ± 1.25	3.25 ± 0.71	2.63 ± 0.52
	LDH(U l^{-1})	197.63 ± 101.78	197.87 ± 68.4	152.5 ± 66.72	262 ± 136.03	158.63 ± 108.83	158 ± 65.04
	CK(U l^{-1})	330.75 ± 57.24	310.25 ± 55.11	296.75 ± 99.87	270.63 ± 88.1	240.63 ± 59.53	226.25 ± 69.45*
	GLU(μmol l^{-1})	6.02 ± 0.34	5.7 ± 0.17	5.93 ± 0.45	5.59 ± 0.4	5.86 ± 0.4	5.83 ± 0.54
	UREA(mmol l^{-1})	6.62 ± 1.5	5.72 ± 1.07	6.62 ± 1.28	5.2 ± 1.54	5.2 ± 1.4	4.63 ± 1.13*
	UA(μmol l^{-1})	12.47 ± 2.77	11.02 ± 1.47	9.53 ± 2.08	10.23 ± 3.08	11.38 ± 2.79	12.71 ± 4.04
	CREA(mmol l^{-1})	81.48 ± 15.05	76.65 ± 8.9	77.41 ± 11.58	79.49 ± 12.3	72.2 ± 8.89	66.84 ± 4.67
	CHOL(mmol l^{-1})	3.92 ± 0.92	3.73 ± 0.97	3.81 ± 0.64	4.02 ± 0.46	3.81 ± 0.69	4.49 ± 0.61
	TG(μmol l^{-1})	0.52 ± 0.11	0.43 ± 0.11	0.52 ± 0.1	0.57 ± 0.1	0.43 ± 0.1	0.49 ± 0.13
	Ca(mmol l^{-1})	2.9 ± 0.09	2.84 ± 0.1	2.87 ± 0.05	2.81 ± 0.06	2.81 ± 0.09	2.81 ± 0.1
	Na(mmol l^{-1})	144.87 ± 2.47	145.25 ± 1.83	146.13 ± 2.17	145.63 ± 1.77	145.38 ± 0.52	146.87 ± 0.99
	K(mmol l^{-1})	5.2 ± 0.33	5.09 ± 0.3	4.99 ± 0.39	5 ± 0.32	5.05 ± 0.44	5.24 ± 0.42
	Cl(mmol l^{-1})	109.88 ± 1.25	110.75 ± 1.67	111 ± 1.2	110.5 ± 1.41	110 ± 1.2	111 ± 1.2
D49 n = 4	ALT(U l^{-1})	45 ± 9.2	37 ± 9.02	42.5 ± 11	42.5 ± 9.15	38.75 ± 2.99	35.25 ± 7.85
	AST(U l^{-1})	42.75 ± 6.55	47.75 ± 8.85	45.25 ± 7.27	45 ± 6.32	40 ± 4.24	40 ± 5.23
	TP(g l^{-1})	69.77 ± 4.65	66.5 ± 1.87	67.4 ± 3.28	71.82 ± 5.89	71.1 ± 3.77	68.1 ± 3.42
	ALB(g l^{-1})	33.65 ± 1.41	33.35 ± 0.86	33 ± 2.74	32.15 ± 0.86	32.03 ± 2.33	32.72 ± 1.15
	GLB(g l^{-1})	36.13 ± 4.53	33.15 ± 2.05	34.4 ± 2.6	39.68 ± 5.96	39.08 ± 5.98	35.38 ± 2.5
	A/G	0.88 ± 0.13	0.98 ± 0.1	0.93 ± 0.1	0.8 ± 0.14	0.77 ± 0.17	0.88 ± 0.05
	TBIL(μmol l^{-1})	2.92 ± 0.13	3.09 ± 0.24	3.21 ± 0.38	3.43 ± 0.25*	1.95 ± 0.26**	2.14 ± 0.17**
	ALP(U l^{-1})	107.5 ± 20.57	103 ± 20.51	93 ± 46.3	105.25 ± 12.12	111.5 ± 61.98	89 ± 18.35
	GGT(U l^{-1})	3 ± 0.82	3 ± 0	3.25 ± 0.5	3 ± 0	3.75 ± 0.5	3.25 ± 0.5
	LDH(U l^{-1})	142.5 ± 123.78	186.75 ± 84.18	172 ± 79.8	176.25 ± 52.61	178 ± 70.1	191 ± 58.26
	CK(U l^{-1})	285.75 ± 72.7	313 ± 47.73	295 ± 21.89	268 ± 27.99	249.75 ± 25.2	256.25 ± 26.74
	GLU(μmol l^{-1})	5.89 ± 0.98	6.2 ± 0.52	6.26 ± 0.28	6.03 ± 0.58	6.13 ± 0.41	5.97 ± 0.36
	UREA(mmol l^{-1})	6.11 ± 1.76	6.02 ± 1.72	6.35 ± 0.9	5.25 ± 0.59	6.88 ± 1.54	6.68 ± 0.77
	UA(μmol l^{-1})	14.2 ± 3.69	13.1 ± 2.39	12.45 ± 2.78	11.8 ± 2.41	14.13 ± 3.07	12.2 ± 4.39
	CREA(mmol l^{-1})	101.3 ± 10.77	97.85 ± 5.45	100.8 ± 11.18	102.28 ± 8.28	64.43 ± 4.34*	59 ± 4.08*
	CHOL(mmol l^{-1})	4.21 ± 1.18	3.93 ± 1.48	3.62 ± 0.6	3.67 ± 0.46	3.69 ± 0.68	4.16 ± 0.65
	TG(μmol l^{-1})	0.56 ± 0.2	0.49 ± 0.16	0.4 ± 0.06	0.58 ± 0.18	0.48 ± 0.09	0.48 ± 0.17
	Ca(mmol l^{-1})	3.02 ± 0.06	2.93 ± 0.03	2.84 ± 0.1**	2.89 ± 0.08	2.89 ± 0.07	2.93 ± 0.04
	Na(mmol l^{-1})	145.25 ± 0.5	146.5 ± 1.91	145.75 ± 0.5	145.5 ± 1.91	144.75 ± 0.96	145 ± 0.82
	K(mmol l^{-1})	5.53 ± 0.76	5.05 ± 0.39	4.8 ± 0.67	4.97 ± 0.46	5.23 ± 0.44	5.18 ± 0.15
	Cl(mmol l^{-1})	109.5 ± 0.58	110.25 ± 1.26	111 ± 0.82	110.5 ± 1	110 ± 1.63	110 ± 0

[a] Values are presented as mean ± SD. *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

The mean concentration-time curves showed that exposure to the DVDMS increased as the dose level increased from 1 to 9 mg kg^{-1} in the treatment groups both with and without PDT after the first dose (Day 1), as well as the last dose (Day 29) (Fig. 4 and 5). Comparing the maximum plasma concentrations between the first cycle and the last treatment cycle, the concentration of DVDMS did not lead to any consistent changes (Fig. 6). The AUC_{0-t} after the last dose was lower than that after the first dose (Fig. 7).

The elimination half-life ($T_{1/2}$) decreased as the dose level increased from 1 mg kg^{-1} to 9 mg kg^{-1} with exposure to DVDMS. Accordingly, the $T_{1/2}$ of DVDMS decreased in each of the high-, medium-, and low-dose groups, and in both the high- and low-dose PDT groups, after five consecutive injections of the test compound (Day 29) compared to that after the first dose (Day 1) (Table 7).

During the first sampling interval, the mean C_{max} and AUC_{0-t} values increased in ratios of 1 : 6.74 : 21.73 and 1 : 6.02 : 21.40, respectively, for a 1 : 3 : 9-fold increase in the dose. During the second sampling interval of toxicokinetics, the mean C_{max} and AUC_{0-t} values increased in a ratio of 1 : 5.25 : 12.00 and 1 : 2.78 : 10.67, respectively, for a 1 : 3 : 9-fold increase in the dose.

After the first administration (Day 1), the 1 mg kg^{-1} DVDMS dose group to 1 mg kg^{-1} DVDMS dose with PDT group ratios of mean C_{max} and AUC_{0-t} were 1 : 1.01 and 1 : 0.91, respectively; after 5 intravenous injections of DVDMS (Day 29), the DVDMS dose group to DVDMS dose with PDT group ratios of

Table 4 Blood coagulation parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$)[a]

		Dose (mg kg^{-1})					
Date	Parameters	0	1	3	9	1 + PDT	9 + PDT
D35 n = 8	TT(s)	14.79 ± 1.05	14.79 ± 0.61	15.04 ± 1.27	18.4 ± 1.13**	13.05 ± 2.72	16.64 ± 2.56
	PT(s)	7.98 ± 0.56	8.13 ± 0.59	7.95 ± 0.62	8.1 ± 0.95	9.48 ± 0.43**	10.07 ± 0.31**
	APTT(s)	9.84 ± 1.37	9.13 ± 0.59	10.19 ± 0.82	10.41 ± 1.15	10.39 ± 0.59	11.74 ± 0.81**
	Fib(g l^{-1})	2.51 ± 0.94	2.32 ± 0.54	2.66 ± 0.81	2.71 ± 0.44	3 ± 0.7	2.79 ± 0.43
D49 n = 4	TT(s)	13.85 ± 1.28	12.93 ± 1.15	13.77 ± 1.1	12.73 ± 0.92	15.2 ± 1.56	15.18 ± 0.46
	PT(s)	8.4 ± 0.72	8.13 ± 0.38	9.05 ± 0.54	8.38 ± 0.17	8.95 ± 0.44	8.98 ± 0.29
	APTT(s)	12.03 ± 0.51	11.25 ± 0.45	9.68 ± 0.53**	11.45 ± 0.62	10.23 ± 0.71**	10.7 ± 0.42*
	Fib(g l^{-1})	2 ± 0.7	2.62 ± 0.35	2.9 ± 0.49	2.96 ± 0.17	2.55 ± 0.37	2.42 ± 0.69

[a] Values are presented as mean ± SD. *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

Table 5 Electrocardiography parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$)[a]

		Dose (mg kg^{-1})					
Date	Parameters	0	1	3	9	1 + PDT	9 + PDT
D35 n = 8	Heart rate	154 ± 37	147 ± 25	143 ± 18	145 ± 19	137 ± 23	150 ± 27
	PR	0.108 ± 0.018	0.11 ± 0.006	0.099 ± 0.012	0.1 ± 0.008	0.1 ± 0.01	0.095 ± 0.007
	QRS	0.045 ± 0.004	0.045 ± 0.003	0.043 ± 0.003	0.042 ± 0.007	0.038 ± 0.004**	0.039 ± 0.004*
	QT	0.171 ± 0.019	0.176 ± 0.009	0.18 ± 0.014	0.171 ± 0.019	0.176 ± 0.011	0.168 ± 0.016
D49 n = 4	Heart rate	149 ± 14	149 ± 38	127 ± 26	150 ± 24	141 ± 28	147 ± 10
	PR	0.108 ± 0.011	0.104 ± 0.006	0.095 ± 0.011	0.103 ± 0.013	0.102 ± 0.009	0.096 ± 0.002
	QRS	0.04 ± 0.004	0.041 ± 0.003	0.039 ± 0.005	0.042 ± 0.007	0.039 ± 0.004	0.036 ± 0.001
	QT	0.172 ± 0.014	0.17 ± 0.01	0.181 ± 0.01	0.166 ± 0.021	0.179 ± 0.005	0.169 ± 0.01

[a] Values are presented as mean ± SD, *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

mean C_{max} and AUC_{0-t} were 1 : 0.81 and 1 : 0.91, respectively, at the dose level of 1 mg kg^{-1} DVDMS. After the first dose of 9 mg kg^{-1} DVDMS (Day 1), the dose group to dose with PDT group ratios of C_{max} and AUC_{0-t} were 1 : 0.89 and 1 : 0.96, respectively, while after injecting 9 mg kg^{-1} DVDMS intravenously 5 times (Day 29), the dose group to dose with PDT group ratios of C_{max} and AUC_{0-t} were 1 : 1.13 and 1 : 1.23.

After 5 successive injections of DVDMS (Day 29), the accumulation coefficients in the 1 mg kg^{-1} (without laser), 3 mg kg^{-1} (without laser), 9 mg kg^{-1} (without laser), 1 mg kg^{-1} (with laser) and 9 mg kg^{-1} (with laser) groups were 1.52, 0.70, 0.76, 1.52 and 0.97, respectively.

3.5 Necropsy and histopathological examination

3.5.1 Organ weight. The absolute and relative organ weights (organ-to-terminal body weight ratio) are summarized in Tables 8–11 for males and females, respectively. In females, the absolute and relative organ weights of the spleen were observed to be significantly higher in the 9 mg kg^{-1} DVDMS-based PDT group on Day 35 and in the 9 mg kg^{-1} DVDMS without illumination group on Day 49, when compared with the negative control group. There were no obviously significant variations in any other organ weights of female dogs in the treatment groups. In males, no statistical differences were observed in absolute or relative organ weights between each dosage group and the saline control group.

3.5.2 Gross examination. Animal euthanasia was carried out at the endpoint of the study, and then a gross anatomy examination was conducted on half of the animals. Discrete, 2–3 mm diameter, pinpoint-shaped, circular, yellow and hard spots were observed on the surface of both kidneys in one dog in the high-dose without PDT group. Decreases in the volumes of the bilateral testes and epididymides were observed in 5 dogs in the high-dose without PDT group, and two of them showed a decline in prostate size. Furthermore, in the high-dose without PDT group, the livers and spleens of two animals became dark red-black. In the high-dose-PDT group, the local skin of five dogs exposed to laser irradiation was damaged and inflamed, and skin crusting was observed at the irradiation site of the skin on another dog.

At the end of the recovery period, there existed some abnormalities in the viscera of several individual animals. For

Table 6 Urinalysis parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$)[a]

Date	Parameters	Dose (mg kg^{-1})					
		0	1	3	9	1 + PDT	9 + PDT
	Sample color/ transparency	4/4 (yellow transparent)	4/4 (yellow transparent)	4/4 (yellow transparent)	4/4 (yellow transparent)	4/4 (yellow transparent)	4/4 (yellow transparent)
D35 n = 4	SG	1.01 ± 0.004	1.006 ± 0.003	1.009 ± 0.005	1.01 ± 0.004	1.016 ± 0.006	1.007 ± 0.005
	LEU	4/4(−)	4/4(−)	4/4(−)	3/4(−) 1/4(+)	4/4(−)	4/4(−)
	NIT	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	pH	7.3 ± 0.5	7.3 ± 1	7.5 ± 0.6	7 ± 0.8	5.8 ± 0.5**	7.3 ± 0.5
	ERY	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	PRO	1/4[(+)] 3/4(−)	1/4[(+)] 3/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	GLU	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	ASC	3/4(++) 1/4(+)	2/4(++) 2/4(+)	4/4(++)	1/4(++) 3/4(+)	3/4(++) 1/4(+)	3/4(++) 1/4(+)
	KET	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	UBG	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	BIL	2/4(+) 2/4(−)	4/4(−)	1/4(+) 3/4(−)	3/4(+) 1/4(−)	2/4(+) 2/4(−)	4/4(−)
	Sample color/ transparency	4/4 (yellow transparent)	4/4 (yellow transparent)	3/4 (yellow transparent) 1/4 (brown turbid)	4/4 (yellow transparent)	4/4 (yellow transparent)	4/4 (yellow transparent)
D49 n = 4	SG	1.007 ± 0.003	1.004 ± 0.005	1.01 ± 0.007	1.01 ± 0	1.016 ± 0.009	1.013 ± 0.009
	LEU	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	NIT	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	pH	7.5 ± 0.6	8 ± 0.8	7.3 ± 1	7 ± 0	6.8 ± 1	7 ± 0.8
	ERY	4/4(−)	3/4(−) 1/4(+)	3/4(−) 1/4(+++)	4/4(−)	4/4(−)	3/4(−) 1/4(+++)
	PRO	1/4[(+)] 1/4(+) 2/4(−)	2/4(++) 2/4(−)	1/4(+) 3/4(−)	4/4(−)	1/4(+) 3/4(−)	1/4[(+)] 1/4(+) 2/4(−)
	GLU	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	ASC	2/4(++) 2/4(+)	3/4(++) 1/4(+)	3/4(++) 1/4(+)	4/4(++)	4/4(++)	3/4(++) 1/4(+)
	KET	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	UBG	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)	4/4(−)
	BIL	2/4(+) 2/4(−)	1/4(+) 3/4(−)	2/4(+) 2/4(−)	1/4(+) 3/4(−)	1/4(++) 2/4(+) 1/4(−)	2/4(+) 2/4(−)

[a] Values of SG and pH are presented as mean ± SD, other parameters are presented as positive rate (positive degree). *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

Table 7 Toxicokinetic parameters of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x} \pm s$, $n = 8$)[a]

Dose (mg kg^{-1})	Date	$T_{1/2}$ (h)	C_{max} (μg mL^{-1})	AUC_{0-t} (μg h ml^{-1})	$AUC_{0-\infty}$ (μg h ml^{-1})
1	D1	45.14 ± 4.31	7.36 ± 2.19	47.74 ± 12.64	57.42 ± 17.13
	D29	36.54 ± 18.14	12.32 ± 2.37	72.46 ± 36.61	97.93 ± 42.77
3	D1	34.88 ± 1.59	49.63 ± 12.10	287.22 ± 47.02	321.03 ± 55.78
	D29	33.01 ± 10.44	64.63 ± 31.02	201.57 ± 65.31	243.39 ± 88.17
9	D1	31.87 ± 3.95	159.94 ± 18.80	1021.23 ± 164.17	1110.24 ± 182.92
	D29	27.99 ± 17.34	147.81 ± 21.37	772.89 ± 201.96	972.19 ± 169.99
1 + PDT	D1	43.62 ± 5.11	7.46 ± 2.78	43.62 ± 17.53	52.36 ± 19.49
	D29	38.03 ± 10.71	9.99 ± 1.58	66.14 ± 21.85	86.72 ± 23.68
9 + PDT	D1	32.10 ± 3.52	142.38 ± 18.67	984.74 ± 183.24	1080.98 ± 206.97
	D29	28.70 ± 8.78	167.06 ± 22.03	952.39 ± 187.08	1107.32 ± 217.19

[a] Values are presented as mean ± SD, $N = 8$. *Significant difference at $P < 0.05$ level, **Significant difference at $P < 0.01$ level, when compared with the control group.

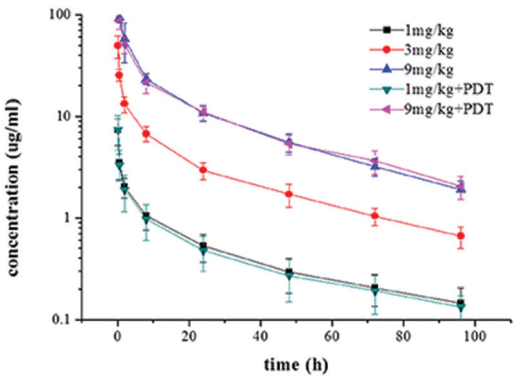

Fig. 4 Mean plasma concentration-time profile of DVDMS in Beagle dogs during the first treatment cycle.

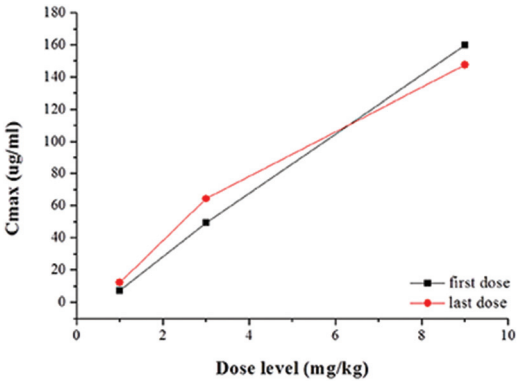

Fig. 6 Maximum plasma concentration-dose level profile of DVDMS in Beagle dogs during the first and last treatment cycle.

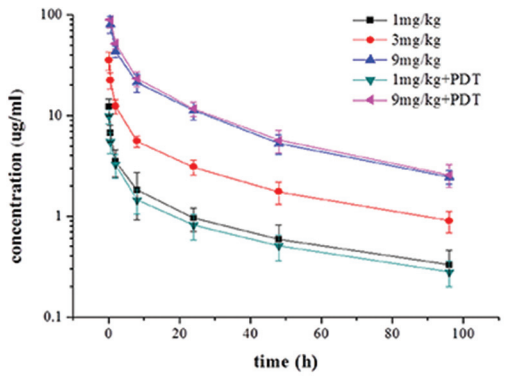

Fig. 5 Mean plasma concentration-time profile of DVDMS in Beagle dogs during the last treatment cycle.

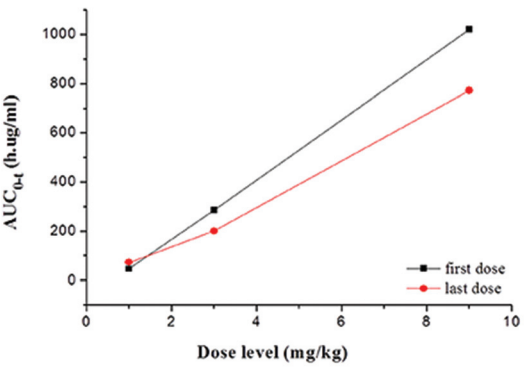

Fig. 7 AUC_{0-4}-dose level profile of DVDMS in Beagle dogs during the first and last treatment cycle.

instance, in the high-dose without PDT group, the liver surface of one dog was brown, the left ventricular wall became thick and the heart cavity shrank. Additionally, it could be observed in one animal from the high-dose without PDT group that the volume of the left kidney was slightly smaller, and the surface of that kidney was uneven; in the same high-dose group without light, the bilateral testes of another dog were small in size.

In the high-dose-PDT group, moreover, the local skin that was exposed to irradiation for one dog appeared exfoliative, red and slightly bulged above the surface. Another two dogs in the high-dose-PDT group showed crusting on the local skin that was illuminated by laser irradiation.

Table 8 Organ weights of female Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x}$, n = 2)[a]

	Dose (mg kg^{-1}) on day 35						Dose (mg kg^{-1}) on day 49					
Organ (♀)	0	1	3	9	1 + PDT	9 + PDT	0	1	3	9	1 + PDT	9 + PDT
Heart	67.43	76.17	68.16	70.00	69.60	80.27	66.65	75.655	77.79	79.61	77.465	76.97
Liver	282.13	253.11	248.70	218.95	234.22	277.56	286.15	294.695	259.03	296.145	295.2	279.985
Spleen	25.91	28.85	30.67	28.99	25.14	**41.95**	29.145	34.27	28.28	**53.425**	27.495	28.425
Lung	74.30	82.11	72.69	77.17	79.10	80.64	74.86	102.035	82.39	90.205	80.33	88.97
Kidney	38.72	49.59	39.96	40.40	41.89	49.24	41.585	48.945	41.785	48.835	41.525	53.74
Adrenal	1.17	1.24	1.34	1.32	1.17	1.22	1.14	1.125	1.435	1.385	1.595	1.16
Thymus	29.97	26.11	24.12	21.32	28.60	20.21	20.08	36.68	22.42	27.38	14.255	29.23
Brain	75.52	78.88	66.90	83.47	77.67	74.31	79.95	81.525	70.42	78.005	86.34	79.495
Overy	0.85	1.10	1.66	0.74	0.80	2.04	0.87	0.955	1.195	2.715	1.6	1.095
Uterus	4.17	3.33	10.49	2.95	2.95	12.51	1.445	3.47	7.48	15.675	10.63	3.34

[a] Values are presented as means, N = 2.

Table 9 Organ weights of male Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x}$, n = 2)[a]

	Dose (mg kg^{-1}) on day 35						Dose (mg kg^{-1}) on day 49					
Organ (♂)	0	1	3	9	1 + PDT	9 + PDT	0	1	3	9	1 + PDT	9 + PDT
Heart	74.34	77.58	67.15	74.68	69.00	68.76	76.78	74.96	60.775	78.985	69.15	76.39
Liver	301.49	286.56	271.67	294.23	252.77	276.80	269.095	259.7	264.02	296.76	267.26	278.01
Spleen	25.13	39.31	25.61	35.31	24.25	27.49	34.83	29.7	27.52	34.75	31.18	27.055
Lung	79.95	95.87	95.14	104.20	80.37	93.40	90.075	86.065	91.265	96.66	92.625	91.825
Kidney	50.65	49.68	41.50	57.82	48.07	43.12	44.865	43.1	39.82	44.82	48.71	42.665
Adrenal	1.02	1.15	1.06	1.30	1.24	1.45	1.475	1.08	1.085	1.19	1.355	1.28
Thymus	28.97	28.38	30.58	24.74	14.96	14.62	23.34	24.395	20.21	20.185	21.22	20.88
Brain	68.21	87.74	73.82	74.75	73.66	83.23	79.635	76.935	76.505	80.15	79.095	83.725
Testis	15.59	11.64	4.60	4.36	13.04	7.29	14.435	11.39	5.02	13.53	16.005	8.095
Epididymis	3.33	3.30	1.43	1.87	2.74	1.82	3.61	2.81	1.805	3.32	4.345	2.05

[a] Values are presented as means, N = 2.

Table 10 Related organ weights (organ-to-terminal body weight ration) of female Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x}$, n = 2)[a]

	Dose (mg kg^{-1}) on day 35						Dose (mg kg^{-1}) on day 49					
Organ (♀)	0	1	3	9	1 + PDT	9 + PDT	0	1	3	9	1 + PDT	9 + PDT
Heart	0.72	0.78	0.74	0.76	0.80	0.83	0.62	0.70	0.77	0.77	0.79	0.75
Liver	3.00	2.60	2.69	2.38	2.69	2.86	2.66	2.73	2.55	2.85	3.01	2.73
Spleen	0.28	0.30	0.33	0.32	0.29	**0.43**	0.27	0.32	0.28	**0.51**	0.28	0.28
Lung	0.79	0.84	0.79	0.84	0.91	0.83	0.70	0.94	0.81	0.87	0.82	0.87
Kidney	0.41	0.51	0.43	0.44	0.48	0.51	0.39	0.45	0.41	0.47	0.42	0.52
Adrenal	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01
Thymus	0.32	0.27	0.26	0.23	0.33	0.21	0.19	0.34	0.22	0.26	0.15	0.29
Brain	0.80	0.81	0.72	0.91	0.89	0.77	0.74	0.75	0.69	0.75	0.88	0.78
Overy	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.03	0.02	0.01
Uterus	0.04	0.03	0.11	0.03	0.03	0.13	0.01	0.03	0.07	0.15	0.11	0.03

[a] Values are presented as means, N = 2.

3.5.3 Histopathological examination. After the final dosing and irradiation (Day 35), slight or mild brown-and-yellow pigmentation was observed in the liver, spleen and local lymph nodes of the 3 mg kg^{-1} DVDMS, 9 mg kg^{-1} DVDMS and 9 mg kg^{-1} DVDMS-based groups. Additionally, the bone marrow of dogs in the 9 mg kg^{-1} DVDMS and 9 mg kg^{-1} DVDMS-based groups showed accumulations of DVDMS pigments (Fig. 8–11). The pathological changes above were not fully restored till the end of the convalescence period (Day 49). There existed no pathological changes in other organs, such as the brain and kidneys, in the 9 mg kg^{-1} DVDMS and 9 mg kg^{-1} DVDMS-based groups (Fig. 12). Moreover, no other

Table 11 Related organ weights (organ-to-terminal body weight ration) of male Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment ($\bar{x}$, n = 2)[a]

	Dose (mg kg^{-1}) on day 35						Dose (mg kg^{-1}) on day 49					
Organ (♂)	0	1	3	9	1 + PDT	9 + PDT	0	1	3	9	1 + PDT	9 + PDT
Heart	0.75	0.81	0.70	0.76	0.78	0.76	0.74	0.73	0.60	0.77	0.69	0.77
Liver	3.05	3.00	2.82	2.99	2.87	3.04	2.60	2.53	2.63	2.88	2.69	2.79
Spleen	0.25	0.41	0.27	0.36	0.28	0.30	0.34	0.29	0.27	0.34	0.31	0.27
Lung	0.81	1.00	0.99	1.06	0.91	1.03	0.87	0.84	0.91	0.94	0.93	0.92
Kidney	0.51	0.52	0.43	0.59	0.55	0.47	0.43	0.42	0.40	0.44	0.49	0.43
Adrenal	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01
Thymus	0.29	0.30	0.32	0.25	0.17	0.16	0.23	0.24	0.20	0.20	0.21	0.21
Brain	0.69	0.92	0.76	0.76	0.84	0.91	0.77	0.75	0.76	0.78	0.79	0.84
Testis	0.16	0.12	0.05	0.04	0.15	0.08	0.14	0.11	0.05	0.13	0.16	0.08
Epididymis	0.03	0.03	0.01	0.02	0.03	0.02	0.03	0.03	0.02	0.03	0.04	0.02

[a] Values are presented as mean, N = 2.

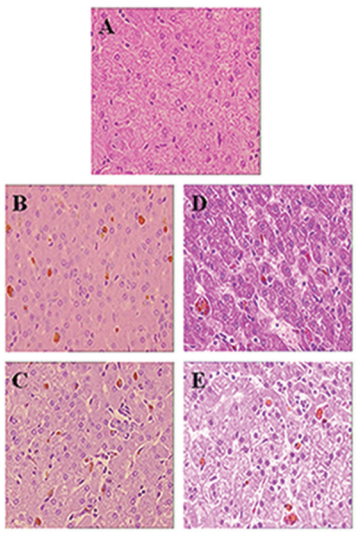

Fig. 8 Light micrograph of liver sections from Beagle dogs. A Control dog. B DVDMS treated dog at 9 mg kg^{-1} (Day 35). C DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 35). D DVDMS treated dog at 9 mg kg^{-1} (Day 49). E DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 49).

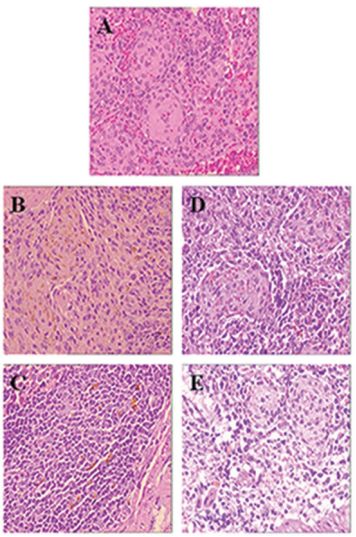

Fig. 9 Light micrograph of spleen sections from Beagle dogs. A Control dog. B DVDMS treated dog at 9 mg kg^{-1} (Day 35). C DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 35). D DVDMS treated dog at 9 mg kg^{-1} (Day 49). E DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 49).

DVDMS-related lesions were observed in the animals of the low-dose or low-dose DVDMS-based PDT group. The degrees of pathological changes and morbidities are shown in Table 12.

The vessels of animals in each treatment group and control group showed no obvious abnormality except that the perivascular tissues of only a few animals showed bleeding and necrosis with inflammatory cell infiltration. At the end of the treatment period (Day 35), slight or mild brown-and-yellow pigmentation could be observed in the perivascular tissues of three dogs and one dog in the 9 mg kg^{-1} DVDMS group and 1 mg kg^{-1} DVDMS-based PDT group, respectively. The same pigmentations were also found in one dog from the 3 mg kg^{-1} and 9 mg kg^{-1} DVDMS groups during the recovery period (Day 49).

At the end of dosing and irradiation, slight to moderate necrosis in the irradiated skin, subcutaneous tissue oedema, fibroblast proliferation associated with bleeding, and a large amount of inflammatory cell infiltration occurred in 3 cases of the 1 mg kg^{-1} DVDMS-based PDT group. In the 9 mg kg^{-1} DVDMS-based PDT group, slight or mild/moderate epidermal necrosis occurred in 2 cases; fibroblast proliferation in subcutaneous tissues occurred in 4 animals; and mild subcutaneous edema was observed in 2 dogs. After the convalescence period (Day 49), in the 1 mg kg^{-1} DVDMS-based PDT group, slight hyperplasia of epidermal cells occurred in 2 cases, and slight fibroblast proliferation in irradiated subcutaneous tissues occurred in 1 animal. At the same stage, 3 cases of slight or mild epidermal necrosis and slight to moderate fibroblast proliferation in the subcutaneous tissues of 4 dogs were observed in the 9 mg kg^{-1} DVDMS-based PDT group.

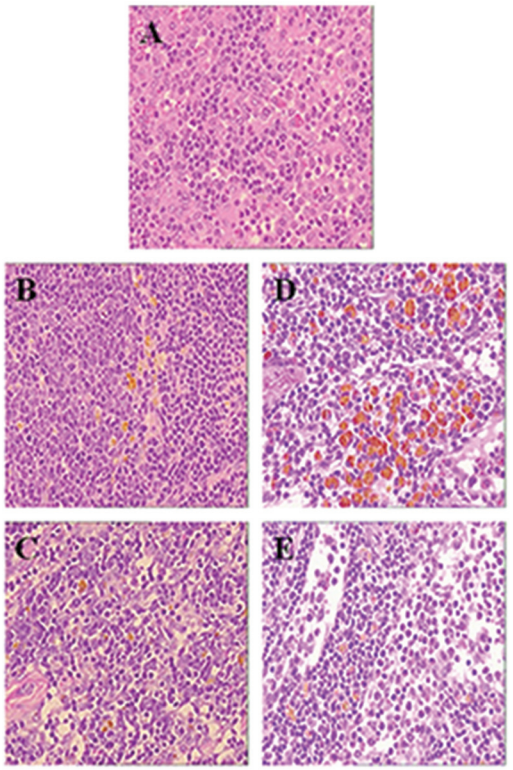

Fig. 10 Light micrograph of local lymph node sections from Beagle dogs. A Control dog. B DVDMS treated dog at 9 mg kg^{-1} (Day 35). C DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 35). D DVDMS treated dog at 9 mg kg^{-1} (Day 49). E DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 49).

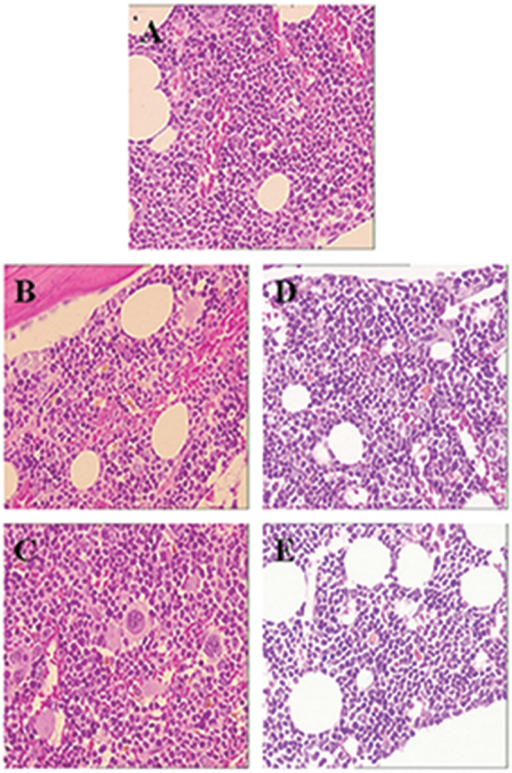

Fig. 11 Light micrograph of marrow sections from Beagle dogs. A Control dog. B DVDMS treated dog at 9 mg kg^{-1} (Day 35). C DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 35). D DVDMS treated dog at 9 mg kg^{-1} (Day 49). E DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 49).

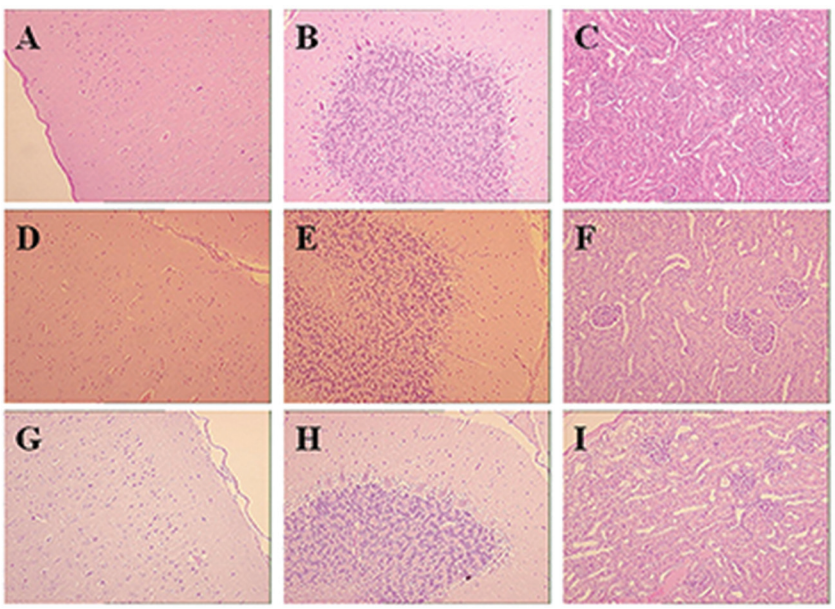

Fig. 12 Light micrograph of brain (cerebrum and cerebellum) and kidney sections from Beagle dogs. A–C cerebrum (A), cerebellum (B) and kidney (C) of control dog (Day 35). D–F cerebrum (D), cerebellum (E) and kidney (F) of DVDMS treated dog at 9 mg kg^{-1} (Day 35). G–I cerebrum (G), cerebellum (H) and kidney (I) of DVDMS treated dog at 9 mg kg^{-1} and followed by illumination (Day 35).

Table 12 Morbidity and lesion degree of organ pigmentation of Beagle dogs exposed to DVDMS intravenously and irradiated with a 630 nm laser light for a 5 week period followed by a subsequent two week recovery period without treatment[a]

		Dose on day 35 (N = 4)						Dose on day 49 (N = 4)					
Organ	Lesion degree	0	1	3	9	1 + PDT	9 + PDT	0	1	3	9	1 + PDT	9 + PDT
Liver	+	0	0	4	0	0	0	0	0	2	0	0	0
	2+	0	0	0	4	0	4	0	0	0	4	0	4
Spleen	+	0	0	2	1	0	0	0	0	1	1	0	2
	2+	0	0	0	3	0	4	0	0	0	3	0	2
Local lymph node	+	0	0	0	1	0	2	0	0	0	1	0	2
	2+	0	0	1	1	0	0	0	0	0	0	0	0
Marrow	+	0	0	0	4	0	3	0	0	0	4	0	4
	2+	0	0	0	0	0	0	0	0	0	0	0	0

[a] Observation by optical microscope. The lesion degrees were divided into four classes: "+" expresses slight, "2+" expresses mild, "3+" expresses moderate, "4+" expresses severe.

4. Discussion

PDT, known as an effective, safe and well-tolerated therapeutic modality for oncotherapy, has been widely used in clinical treatment for decades.[11] Photosensitizers, as a critical factor in PDT, have been well developed and are used in preclinical studies before they are available in the clinical treatment of tumors. Sinoporphyrin sodium (DVDMS), which is isolated from Photofrin II, has the advantages of 98.5% chemical purity, high solubility in water, good targeting and brief skin sensitivity.[23] Additionally, DVDMS showed high tumor-inhibiting rates *in vivo*[21] and potent cytotoxicity on tumor cells *in vitro*.[22]

The current study was performed to clarify the toxicity profile of DVDMS-PDT treatment as an antitumor therapy for Beagle dogs by repeated exposure to DVDMS and laser illumination because no such subchronic toxicity test of DVDMS has been previously conducted. The intravenously repeated administration of the DVDMS preparation at doses of 1, 3 and 9 mg per kg per day, followed with or without photo-irradiation *via* a 630 nm laser light 24 h after administration, was well tolerated and produced no deaths. No other obviously adverse findings were found in clinical observation, except that some dogs in the high-dose DVDMS-based PDT group showed a slight conjunctival redness by ophthalmoscopic examination; however, the full recovery of this symptom in convalescence revealed no toxicological significance. An increase in body temperature was observed in dogs of the mid-dose DVDMS group on Day 35, Day 42 and Day 43. In consideration of the lack of other related clinical observations, the slight but dose independent changes in body temperature at some individual research time-points revealed no toxicological significance.

The values of PT in the two PDT groups, APTT in the high-dose DVDMS-based PDT group and TT in the high-dose DVDMS group, had a tendency to increase after the 5-time treatment period. However, the APTT of the mid-dose group and two PDT groups showed an obvious reduction during the recovery period, which suggested that the coagulation system might be affected by treatment with this preparation. When DVDMS is used in clinical practice, especially in combination with drugs acting on the blood coagulation system, the state of the coagulation system should be monitored, and attention should be paid to the safety of patients who have hematological diseases. A recent study reported that tissue factor (TF), a transmembrane glycoprotein that initiates blood coagulation, is expressed in several tumor types and plays a role in tumor growth. TF, as an anti-tumor target, can be inhibited in the process of PDT. From this perspective, the function of coagulation may be affected by photodynamic effects to some extent.[28] Furthermore, the variation tendency of the coagulation system related indexes between the treatment and recovery periods went in the opposite direction. A possible explanation is the compensatory mechanism and immune regulatory effect on clotting factors in the bodies of the experimented animals, in order to counter the adverse effects induced by the photodynamic effect, and the following abrupt cessation of the DVDMS-PDT treatment.[29]

In addition, the increase in coagulation system parameters might be associated with hepatic dysfunction, and this hypothesis was also corroborated by the significant changes of ALB and A/G in serum biochemistry tests. Recent research has demonstrated that a variety of serum biochemical markers, such as ALB, ALT, PT, APTT and TT, are directly related to liver injury, and these indexes can be used to establish a clinical scoring formula for liver injury.[30] In this study, both the indexes of the coagulation system and liver enzymes showed significant changes. First, the coagulation indexes showed a tendency to increase during the treatment period, while a decreasing tendency was observed in convalescence. This phenomenon might be due to the protective effects of compensatory mechanisms and immune regulation. Additionally, the liver function related parameters (liver enzymes) in the serum biochemistry examination returned to normal during the recovery period. Last but not least, the gross examination showed no obvious pathological change in the liver, while the histopathological examination merely revealed pigmentation on the liver. The above reasons demonstrate that DVDMS-PDT treatment induced a slight, temporary but significant transient change on hepatic function. Nevertheless, the pigmentation in the liver was not completely eliminated during convalescence,

indicating that DVDMS-PDT treatment probably had a long-term effect on the liver. Thus, special attention still needs to be paid to patients who have hepatic dysfunction or liver diseases.

A few minor changes in hematological and other slight variances in the serum biochemistry test and coagulation test were likewise observed. However, these abnormal indexes, recorded after the dogs were administered DVDMS and photodynamic therapy was conducted, indicating no significant difference from the values measured before administration, and the variation of numerical values was within the range of historical control data from our institution. Therefore, we could not conclude that the abnormalities were directly related to DVDMS treatment or DVDMS-based PDT.

The QRS duration of the DVDMS-PDT groups was significantly decreased; however, it indicated no remarkable difference from the numerical value, which was measured before the administration. Accordingly, we considered that the changes observed in the electrocardiography examinations could not be attributed to DVDMS-based PDT related toxicity.

The change in pH value of the 1 mg kg^{-1} DVDMS-PDT group was dose-independent, and other indicators showed no apparent abnormality; thus, we concluded that the variation was not directly related to the DVDMS-based photodynamic therapy. Brown turbid urine and strongly positive ERY were observed in the mid-dose DVDMS group. Considering that this abnormality in urinalysis merely developed during the recovery period, and furthermore, no other statistical differences were observed between other treatment groups and the saline control group, we tend to regard this as being independent of the hemolytic reaction. The abnormality index in the urinalysis was strongly suspected to be due to blood sample contamination resulting from our laboratory manipulation.

The study on the toxicokinetics of DVDMS in dogs provides an insight into the time course of concentrations in different dosages. The *in vivo* process of DVDMS was characterized by a fast distribution phase, followed by a moderately rapid elimination phase, consistent with the characteristics of distribution and elimination in the plasma of rats. Several studies showed that the plasma concentration levels of porphyrin-like photosensitizers rapidly decreased after intravenous injection, exhibiting a bi-exponential decline with a rapid distribution phase followed by a moderately rapid elimination phase.[31,32] In addition, the rapid elimination of DVDMS contributed to a short light-avoidance period. C_{max} and AUC_{0-t} after the first administration increased proportionally with the dose in a linear fashion, and no saturation was observed. The increases in C_{max} and AUC_{0-t} values were approximately proportional to the increase in the dose level during the second collection interval. The systematic exposure of DVDMS in Beagle dogs was in a dose-dependent manner. The $T_{1/2}$ of DVDMS preparation leaked out from the injection site into the perivascular tissue when it was administrated intravenously; thus, the experimental manipulation was the reason for the pigmentation. Therefore, we should pay more attention to prevent leaking out and deposition of the DVDMS preparation around the vessel.

In the previous study, DVDMS showed reduced skin phototoxicity compared to Photofrin® in tumor-bearing mouse models.[21] Similarly, in our research, skin swelling and ulceration were observed in dogs that received PDT during the treatment period, and the ulceration and escharosis of skin did not fully recover until the end of the convalescence period. Additionally, microscopic observation revealed that laser illumination could induce a series of healing responses on animals at the end of treatment, such as skin necrosis, edema, fibroblast proliferation secondary to inflammatory-cell infiltration and hyperplasia of epidermal cells. The irradiated skin was not totally restored 2 weeks after the termination of treatment. Thus, slight but somehow continuous phototoxic reaction of the skin of dogs is consistent with the response observed in previous reports. Photosensitivity reactions, consisting of phototoxicity and photoallergy, are one of the most prevalent adverse effects of PDT.[33] Clinical trials found that short-term (such as cutaneous photosensitivity and dermatitis) and long-term (such as pigmentation and scar formation) post-treatment responses, induced by porphyrin-like photosensitizer-based PDT, were generally caused by phototoxicity. Therein, edema and scabbing were the most common responses to phototoxicity related to both the photosensitizer and laser exposure.[34] In order to reduce the adverse effects on patients, we can optimize the laser dose in the clinical application of DVDMS-PDT according to the data of preclinical studies on animals. The conditions of cutaneous changes must be monitored, and treatment parameters should be selected for each individual patient when conducting the illumination treatment.

There were a few or several animals whose organs had pathological changes in both of the treatment groups and the control group, and the lesions mostly occurred in the viscera and tissues, such as the heart, liver, kidneys, lungs and pituitary gland. The main manifestations were focally inflammatory cell infiltration in myocardial and/or pulmonary and/or nephritic interstitial tissue, as well as in liver tissue. Similarly, renal tubular dilatation, chronic kidney disease, pituitary gland cysts and male reproductive system dysplasia were observed. The pathological changes above were characterized as being in a slight or mild degree, having a low incidence, showing no differences in incidence and lesion degree among groups, as well as being disperse in occurrence. Accordingly, these changes could be referred to as spontaneous pathological changes, which are commonly seen in Beagle dogs.[35] It is worth noting that, under microscopic observation, slight or mild brown-and-yellow pigmentation of DVDMS (or its metabolite) granules was observed in the liver, spleen, local lymph nodes and bone marrow of mid-dose, high-dose, and high-dose-PDT groups. The deposit of DVDMS pigment was not eliminated till the end of the convalescence. Moreover, in the gross examination, we observed in some dogs that the color of liver and spleen became dark to some extent. This apparent pigmentation effect provides a clue as to how the granules of

DVDMS are distributed and metabolized *in vivo*. Studies have shown that the main locations of metabolism of porphyrin-like photosensitizers are the liver and spleen.[36,37] Considering that the porphyrin-like photosensitizers are retained longer in the liver, spleen and kidneys, patients with severe liver or kidney disease should avoid PDT.[38]

We observed that the pigmentation of DVDMS focused on the immune tissue, such as the spleen, lymph nodes and bone marrow. Moreover, pigmentation of the immune tissue could also be observed in several dogs through gross pathological examination, which was manifested as the color of spleen being darker to some degree. In conjunction with the pathological changes in immune tissue, the organ weights (absolute and relative) of the spleen in female dogs treated with high-dose DVDMS and illumination showed a significant increase. Similarly, the phenomenon of pigmentation in bone marrow and the changes in blood coagulation indicate that PDT may have an association with the regulation of the immune system. Pre-clinical and clinical studies have demonstrated that PDT is capable of stimulating both the innate and adaptive immune responses, which induce the release of pro-inflammatory molecules, generating tumor-specific cytotoxic T-cells capable of destroying distant untreated tumor cells, and leading to the development of anti-tumor memory immunity that can potentially prevent the recurrence of cancer.[39] Studies showed that mice whose tumors had been eliminated by PDT were resistant to subsequent tumor challenges, and tumor draining lymph node cells isolated from PDT-treated mice are able to transfer tumor immunity to naive mice.[40] Recently, photoimmunotherapy (PIT) has become a research topic of interest, such as PDT in combination with immunostimulants.[41,42] Additionally, another promising option is anticancer vaccination using immature DCs that have been treated with PDT.[43]

5. Conclusions

In conclusion, following intravenous injection of DVDMS and photodynamic therapy in Beagle dogs for 30 days, the viscera of the animals showed no obvious treatment-related toxicity. However, there still existed, to some extent, phototoxic reactions of the irradiated skin. Slight or mild pigmentation was observed in several tissues (including the liver, spleen, local lymph nodes and marrow). The pathological changes above did not completely resume during 2 weeks after withdrawal of DVDMS. Additionally, the variances in blood coagulation and serum biochemistry parameters provide clues that the main metabolism locus and toxic target organs are the liver and spleen. Furthermore, the results of this study showed a great correlativity between PDT and immune regulation, which is consistent with the viewpoints of photosensitizer-antibody conjugates for the detection and therapy of cancer. In toxicokinetic studies, no delayed or accumulative pathological changes were found. Intravenous injection of DVDMS in Beagle dogs revealed no vascular irritation. The NOAEL was considered to be 1 mg kg^{-1}, and DVDMS-PDT appeared to be a safe and promising therapeutic modality for tumor treatment.

Our further work will be designed to investigate if the variances of serum biochemistry, blood coagulation and pathology have association with the effects on liver function induced by DVDMS, by means of detecting more liver function indices of animals repeatedly exposed to DVDMS. Additionally, pharmacological research on the immune response induced by DVDMS-PDT is being carried out in our institute. Thus, we plan to conduct immunotoxicity studies on DVDMS as the foundation of the ongoing study, in order to determine if there is any adverse effect on the structure or function of the immune system. Moreover, considering the inevitability of light-induced injury in the process of PDT, trials aiming at optimizing the light dose will be performed to reduce the suffering of cancer patients.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

1 T. Luo, B. C. Wilson and Q. B. Lu, Evaluation of one- and two-photon activated photodynamic therapy with pyropheophorbide-a methyl ester in human cervical, lung and ovarian cancer cells, *J. Photochem. Photobiol., B*, 2014, **132**, 102–110.
2 T. Yano, K. Hatogai, H. Morimoto, Y. Yoda and K. Kaneko, Photodynamic therapy for esophageal cancer, *Ann. Transl. Med.*, 2014, **2**, 29.
3 K. H. Nelke, W. Pawlak, J. Leszczyszyn and H. Gerber, Photodynamic therapy in head and neck cancer, *Postepy Hig. Med. Dosw.*, 2014, **68**, 119–128.
4 J. Neuhaus, S. Schastak, M. Berndt, J. Walther, A. Dietel, N. Sieger and J. U. Stolzenburg, Photodynamic therapy of bladder cancer. A new option, *Urologe A*, 2013, **52**, 1225–1232.
5 V. Monge-Fuentes, L. A. Muehlmann and R. B. de Azevedo, Perspectives on the application of nanotechnology in photodynamic therapy for the treatment of melanoma, *Nano Rev.*, 2014, **5**, 1–14.

6 A. Chwilkowska, J. Kulbacka and J. Saczko, Death of tumor cells. Photodynamic reaction in apoptosis induction in cancer cells, *Pol. Merkuriusz Lek.*, 2011, **30**, 45–48.
7 J. O. Yoo and K. S. Ha, New insights into the mechanisms for photodynamic therapy-induced cancer cell death, *Int. Rev. Cell Mol. Biol.*, 2012, **295**, 139–174.
8 P. Skupin-Mrugalska, L. Sobotta, M. Kucinska, M. Murias, J. Mielcarek and N. Duzgunes, Cellular changes, molecular pathways and the immune system following photodynamic treatment, *Curr. Med. Chem.*, 2014, **21**, 4059–4073.
9 M. Y. Lee, W. K. Lee, J. Baek, O. W. Kwon and J. H. Lee, Photodynamic therapy versus combination therapy in polypoidal choroidal vasculopathy: changes of aqueous vascular endothelial growth factor, *Am. J. Ophthalmol.*, 2013, **156**, 343–348.
10 N. Shirasu, S. O. Nam and M. Kuroki, Tumor-targeted photodynamic therapy, *Anticancer Res.*, 2013, **33**, 2823–2831.
11 M. A. MacCormack, Photodynamic therapy, *Adv. Dermatol.*, 2006, **22**, 219–258.
12 H. Kato, History of photodynamic therapy–past, present and future, *Gan to Kagaku Ryoho*, 1996, **23**, 8–15.
13 Y. K. Ho, R. K. Pandey, J. R. Missert and T. J. Dougherty, Some components of the tumor-localizing fraction of hematoporphyrin derivative, *Photochem. Photobiol.*, 1990, **52**, 1085–1088.
14 M. Wainwright, Photodynamic therapy: the development of new photosensitisers, *Anti-Cancer Agents Med. Chem.*, 2008, **8**, 280–291.
15 R. R. Allison, Photodynamic therapy: oncologic horizons, *Future Oncol.*, 2014, **10**, 123–124.
16 C. J. Byrne, L. V. Marshallsay and A. D. Ward, The composition of Photofrin II, *J. Photochem. Photobiol., B*, 1990, **6**, 13–27.
17 M. J. Garland, C. M. Cassidy, D. Woolfson and R. F. Donnelly, Designing photosensitizers for photodynamic therapy: strategies, challenges and promising developments, *Future Med. Chem.*, 2009, **1**, 667–691.
18 J. D. Breskey, S. E. Lacey, B. J. Vesper, W. A. Paradise, J. A. Radosevich and M. D. Colvard, Photodynamic therapy: occupational hazards and preventative recommendations for clinical administration by healthcare providers, *Photomed. Laser Surg.*, 2013, **31**, 398–407.
19 Q. C. Fang and D. Yang. A porphyrin dimer combined with and ether bond and its manufacturing method, *Patent ZL* 200910179116.5, 2012, People's Republic of China.
20 Q. C. Fang, Photodynamic therapy for cancer treatment and the new antitumor photosensitizer sinoporphyrin sodium, *Chin. J. New Drugs*, 2014, **23**, 1540–1545.
21 Z. H. Jiang, Inhibitory effects of DVDMS-2-based-photodynamic therapy on the growth of tumor and in vitro in vivo, *Teratog., Carcinog., Mutagen.*, 2013, **25**, 163–167.
22 J. Hu, X. Wang, Q. Liu, K. Zhang, W. Xiong, C. Xu, P. Wang and A. W. Leung, Antitumor Effect of Sinoporphyrin Sodium-Mediated Photodynamic Therapy on Human Esophageal Cancer Eca-109 Cells, *Photochem. Photobiol.*, 2014, **90**, 1404–1412.
23 H. Wang, X. Wang, S. Zhang, P. Wang, K. Zhang and Q. Liu, Sinoporphyrin sodium, a novel sensitizer, triggers mitochondrial-dependent apoptosis in ECA-109 cells via production of reactive oxygen species, *Int. J. Nanomed.*, 2014, **9**, 3077–3090.
24 J. Hu, X. Wang, K. Zhang, P. Wang, X. Su, Y. Li, Z. Huang and Q. Liu, Sinoporphyrin sodium: a novel sensitizer in sonodynamic therapy, *Anti-cancer Drugs*, 2014, **25**, 174–182.
25 C. Li, K. Zhang, P. Wang, J. Hu, Q. Liu and X. Wang, Sonodynamic antitumor effect of a novel sonosensitizer on S180 solid tumor, *Biopharm. Drug Dispos.*, 2014, **35**, 50–59.
26 M. Triesscheijn, P. Baas, J. H. Schellens and F. A. Stewart, Photodynamic therapy in oncology, *Oncologist*, 2006, **11**, 1034–1044.
27 W. Clark, M. D. Heath, A. Geneva and S. B. Daland, Staining of reitculocytes with Brilliant Cresly Blue, *Arch. Int. Med.*, 1931, **48**, 133–145.
28 M. Cole and M. Bromberg, Tissue factor as a novel target for treatment of breast cancer, *Oncologist*, 2013, **18**, 14–18.
29 N. Sengul, S. Demirer, M. A. Yerdel, G. Terzioglu, B. Akin, A. Gurler and A. Tuzuner, Comparison of coagulation parameters for healthy subjects and Behcet disease patients with and without vascular involvement, *World J. Surg.*, 2000, **24**, 1584–1588.
30 W. Liu, J. Zheng and R. Xing, Clinical significance of a scoring formula of liver injury for the preoperative evaluation of patients with liver cirrhosis, *Eur. J. Gastroenterol. Hepatol.*, 2014, **26**, 95–100.
31 P. H. Sun, X. Zhao, Y. Zhou, Y. Liang, H. L. Zhang, Y. M. Cui and J. N. Tao, Tolerance and pharmacokinetics of single-dose intravenous hemoporfin in healthy volunteers, *Acta Pharmacol. Sin.*, 2011, **32**, 1549–1554.
32 B. Q. Li, S. H. Fang, X. Dong, N. Li, J. Y. Gao, G. Q. Yang, X. C. Gong, S. J. Wang and F. S. Wang, Pharmacokinetics, tissue distribution and excretion of manganese (III) meso-tetra [3-(2-(2-methoxy)-ethoxy) ethoxy] phenyl porphyrin chloride, a novel superoxide dismutase mimic, in Wistar rats, *Eur. J. Drug Metab. Pharmacokinet.*, 2013, **38**, 245–253.
33 J. H. Epstein, Phototoxicity and photoallergy, *Semin. Cutaneous Med. Surg.*, 1999, **18**, 274–284.
34 K. H. Yuan, J. H. Gao and Z. Huang, Adverse effects associated with photodynamic therapy (PDT) of port-wine stain (PWS) birthmarks, *Photodiagn. Photodyn. Ther.*, 2012, **9**, 332–336.
35 Y. Oghiso, S. Fukuda and H. Iida, Histopathological studies on distribution of spontaneous lesions and age changes in the beagle, *Nihon Juigaku Zasshi. The Japanese Journal of Veterinary Science*, 1982, **44**, 941–950.
36 F. Koszo, C. Siklosi and N. Simon, Effects of iron-loading and ethanol treatment on rat porphyrin metabolism, *Pharmacol., Biochem. Behav.*, 1980, **13**, 325–329.
37 A. Ferrario and C. J. Gomer, Systemic toxicity in mice induced by localized porphyrin photodynamic therapy, *Cancer Res.*, 1990, **50**, 539–543.

38 P. G. Calzavara-Pinton, R. M. Szeimies, B. Ortel and C. Zane, Photodynamic therapy with systemic administration of photosensitizers in dermatology, *J. Photochem. Photobiol., B*, 1996, **36**, 225–231.
39 E. Reginato, P. Wolf and M. R. Hamblin, Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects, *World J. Immunol.*, 2014, **4**, 1–11.
40 S. O. Gollnick, B. Owczarczak and P. Maier, Photodynamic therapy and anti-tumor immunity, *Lasers Surg. Med.*, 2006, **38**, 509–515.
41 N. P. Brodin, C. Guha and W. A. Tome, Photodynamic Therapy and Its Role in Combined Modality Anticancer Treatment, *Technol. Cancer Res. Treat.*, 2014, DOI: 10.1177/1533034614556192.
42 E. J. Sanchez-Barcelo and M. D. Mediavilla, Recent patents on light based therapies: photodynamic therapy, photothermal therapy and photoimmunotherapy, *Recent Pat. Endocr. Metab. Immune Drug Discovery*, 2014, **8**, 1–8.
43 Y. Zheng, G. Yin, V. Le, A. Zhang, Y. Lu, M. Yang, Z. Fei and J. Liu, Hypericin-based Photodynamic Therapy Induces a Tumor-Specific Immune Response and an Effective DC-based cancer Immunotherapy, *Biochem. Pharmacol.*, 2014, DOI: 10.1016/j.bcp.2014.01.036.

