Vascular-targeted photodynamic therapy (VTP) of a canine-transmissible venereal tumour in a murine model with Pd-bacteriopheophorbide (WST09)

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Abstract

Treatment of canine-transmissible venereal tumour (CTVT) with local vascular-targeted photodynamic therapy (VTP) using Pd-bacteriopheophorbide (WST09) as a drug is suggested as an alternative to conventional chemotherapy. Male CD1 nude mice were subcutaneously grafted with the xenograft-transmissible canine venereal tumour (XTVT). The VTP protocol delivered once consisted of intravenous administration of WST09 (10 mg kg^{-1}) followed by immediate local illumination with a diode laser (763 nm). Controls included animals treated with light or WST09 alone. Macroscopic and microscopic evaluations of tumour response were conducted 10, 24 and 48 h after treatment. Upon VTP, tumours underwent necrosis that lasted 8–10 days and exhibited complete healing by 25–35 days, reaching an overall long-term cure rate (83%) by 90 days after treatment. This study suggests that VTP with WST09 can efficiently treat CTVT in a single session, as compared with 4–6 sessions of chemotherapy and thus may be feasible for common veterinary practice, particularly under ambulatory conditions.

Keywords
antivascular therapy, canine-transmissible venereal tumour (CTVT), Pd-bacteriopheophorbide, photodynamic therapy, WST09

Introduction

Canine-transmissible venereal tumour (CTVT) of dogs is usually a coitally transmitted neoplasm of the external genitalia. The tumour has been reported to be endemic in temperate climates in various parts of the world. CTVT is transmitted by implantation of cells during coitus[1–3].

The origin of CTVT is not known but believed to be reticuloendothelial. Although virus-like particles have been observed inside tumour cells, CTVT cannot be transmitted by cell-free filtrates[4,5]. Most tumour cells contain 59 chromosomes, while normal dog cells contain 78, but the total amount of DNA is close to normal[2].

The tumour begins as solitary or multiple nodules on the glans penis or bulbus glandis in male and anywhere in the female's vagina. It also may develop on extragenital sites on the skin or around the mouth. Metastases are uncommon but have been reported in the superficial inguinal and external iliac lymph nodes[3]. Tumours have also been reported in the brain, eye, spleen and liver[6–8]. In addition to the natural course of transmission,

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experimental transplantation is well documented in dogs[3]. Heterotransplantation of CTVT has also been attempted. The tumour can be transplanted to foxes, jackals and coyotes[3,9]. Implantation was also successful in irradiated mice and in athymic nude mice[10,11]. We recently reported on xenotransplantation of this tumour in a murine model NOD/LtSz-scid mice (NOD/SCID)[1].

CTVT responds to many forms of therapy: surgery, radiation and chemotherapy. Recurrences up to 60% have been reported after surgery[12]. Radiation therapy is more successful but requires special equipment and trained staff. In a reported case of orthovoltage radiation therapy, three treatment sessions were used to induce complete remission of the tumour[13]. However, as megavoltage treatment is further introduced to veterinary oncology, corresponding new treatment protocols may be required[14]. Chemotherapy with vincristine is an effective mode of CTVT treatment, but Cyclophosphamide, Methotrexate and Doxorubicin are also used. Four to six weekly treatments are required for successful chemotherapy. In spite of positive responses to chemotherapy, cases of partial and non-responding tumours have been reported[15–17].

In the case of resistance to chemotherapy as observed with many other tumour types, indirect targeting by antiangiogenic and antivascular treatments may be considered as alternatives[18]. Indeed, several antiangiogenic agents are now under investigation as anticancer agents that prevent neovascularization[19]. In addition, in antivascular chemotherapy that targets existing tumour blood vessels and leads to their occlusion with consequent hypoxia, necrosis and tumour eradication[18,19].

Photodynamic therapy (PDT) in its classical sense is a mode of cancer therapy in which drug action is locally controlled by light. Illumination of the sensitizer drug in the tumour generates a local burst of cytotoxic reactive oxygen species (ROS) which destroy vital cellular components, inducing tumour cell death and necrosis, while sparing surrounding normal tissue[20–23].

We have synthesized a novel family of photosensitizers, derived from the photosynthetic pigment bacteriochlorophyll[24,25], which effectively eradicates solid tumours, but by an antivascular mechanism. We have successfully used Mg-bacteriochlorophyll serine as PDT agent for treatment of melanoma and DS sarcoma[26–28]. Subsequently, the advanced drug, Pd-bacteriopheophorbide (WST09) was used for photodynamic treatment of xenografts (rat C6 glioma, normal canine prostate, human prostatic small cell carcinoma, human prostatic adenocarcinoma and human HT29 colon carcinoma) in various animal models and in healthy dogs[29–35]. WST09, which is manufactured by Steba Biotech, Paris, France and Negma Lerads, Toussus-Le-Noble, France, has entered phase II clinical trials for prostate cancer therapy in several countries (USA, UK and Israel)[36]. The bacteriochlorophyll-based photosensitizers, including WST09, exhibit advantageous photochemical and pharmacological characteristics to most clinically used photosensitizers: (i) high extinction coefficient in the near infrared (IR) ($\varepsilon_0 \sim 4 \times 10^4$ mol^{-1} cm^{-1} at 763 nm in water) enabling treatment of bulky tumours to a depth of 2 cm[29], (ii) rapid clearance from the circulation (<10 minutes) and skin (no phototoxicity at times >1 h after treatment) minimizing skin phototoxicity (Koudinova, unpublished) and (iii) the PDT treatment protocol is rapid (10 minutes), completed in a single session where the sensitizer and light are sequentially administered with no time interval in laboratory animals or during 20 minutes after drug infusion with overlapping illumination (17 minutes) in humans. As shown by us, this WST09-based vascular-targeted protocol PDT (VTP) selectively induces tumour blood vessel occlusion and stasis within minutes of illumination[30] with consequent local ischaemia, culminating with necrosis (24–48 h) and ultimate tumour eradication in weeks[32]. Most importantly, as WST09-PDT targets the tumour blood vessels. It was found by us to equally efficiently eradicate human multidrug-resistant and wild-type colon HT29 xenografts in mice[34]. The rationale of this new VTP protocol is in its unique principle where the target of treatment is the tumour vasculature and not the tumour cells. Here the sensitizer is exposed to light while still in the blood stream, leading to vascular occlusion, blood stasis and tumour eradication. This process is technically

enabled by concomitant sensitizer injection/infusion and tumour illumination and by minimal extravagation of the drug from the blood vessels[28,30,32]. During the procedure, the illuminated vascular bed of the tumour acts as a flow-through reactor in which oxygenated blood carrying sensitizer is photosensitized to continually generate ROS within the vascular compartment[30]. Thus, solely confined to the light field, the tumour vasculature is selectively destroyed by a maintained cytotoxic cloud of photogenerated ROS and by free-radical chain reactions that persist in the tumour for hours after the light is turned off. The selectivity of this process relies on increased susceptibility of the tumour vasculature when compared with blood vessels in the normal surrounding tissue. This same principle is being successfully used in clinical VTP of non-malignant pathologic choroidal neovasularization in the eye during ophthalmic treatment of age-related macular degeneration[37]. This is in contrast to the common mechanism of PDT, which is based on photosensitization of preaccumulated sensitizer within the target tumour cells directly leading to their death[20–23].

In the present study, we describe a murine model of CTVT and demonstrate its response to WST09-VTP. Although a mouse model was used in the preclinical phase to allow practical examination of a large number of cases, the long-term objective is clinical treatment of canine tumours with WST09-VTP. Indeed, the results suggest that local WST09-VTP is a successful treatment for CTVT, which is applicable under ambulatory conditions, for use in veterinary practice.

Materials and methods

Animals

Male CD1 nude mice (28–32 g) were housed in the Weizmann Institute animal facility, and all animal experiments were conducted according to the guidelines of the institutional animal care and use committee. Fifty-seven mice were used.

Tumour model

CTVT was transferred from intermediate grafting in NOD/LtSz-scid mice, xenograft-transmissible venereal tumour (XTVT)[1]. Small pieces ($\sim 2 \times 2$ mm) were prepared in phosphate-buffered saline (PBS) under sterile conditions. The pieces were implanted subcutaneously on the flank of the mice. After 10–12 weeks, the tumours reached the treatment size (diameter 6–8 mm). Tumour size was measured with a caliper and the volume (mm^3) calculated according to the formula: *length* * *width* * *height* * 0.5236[38]. Animals bearing tumours >600–800 mm^3 were euthanized using standard protocols.

Light source

A 763-nm diode laser with variable output (0.02–1 W) (CERAMOPTEC, Bonn, Germany) equipped with a 0.5-mm diameter light guide (cleaved tip) was used as previously described[30,32,34,35]. The output of the instrument was verified spectroscopically and was found to fit with the factory specifications. The delivered light fluency at the target level was measured independently (using an optical power meter, Ophir Optronics LTD Jerusalem, Israel) and adapted to the programmed light dose per area of selected illuminated target.

Photosensitizer

WST09 (2.5 mg mL^{-1}) was administered in 5% Cremophor El-based formulation STEBA BIOTECH and NEGMA LERADS, France.

Anaesthesia

A mixture of ketamine 100 mg mL^{-1} (Rhone Merieux, Lyon, France) and xylazine 2% (Vitamed, Hadera, Israel) (85:15 v:v) in concentration of 1.5 mL kg^{-1} was intraperitoneally administrated to mice.

VTP protocol

Anaesthetized mice were intravenously injected (tail vein) with WST09 (10 mg kg^{-1}). The tumour was transcutaneously illuminated with no delay (including a 2–3 mm margin of normal surrounding tissue) at 150 mW/cm^2 for 10 minutes (90 J cm^2). After the treatment, the mice were returned to the cage.

Controls

Dark control

The anaesthetized mouse was injected intravenously with WST09 and placed in a dark cage without illumination for 24 h.

Light control

The tumours were illuminated under the same conditions without WST09 treatment.

Treatment end points

Local necrosis at the treatment site was taken as the successful endpoint of treatment. Cure was defined as 'no tumour recurrence' by 90 days post-treatment.

Tumour necrosis

Tumour necrosis was followed and recorded photographically. The tumour volume was calculated as the size of crust, with the same formula.

Histology

Tumours were excised from sacrificed animals, fixed in 4% formaldehyde in PBS at room temperature (RT) (48 h) and paraffin embedded. Sections were prepared and stained with haematoxylin/eosin (H&E) using standard protocols[32]. Samples were taken at 10, 24 and 48 h after VTP from treatment group and 24 h after treatment from control groups. Samples were also taken from untreated mice.

Immunohistochemistry

Paraffin-embedded tumour sections were prepared for immunohistochemistry, and lipid peroxidation (LPO) was monitored using 4-hydroxy-2-nonenal (HNE) as a marker of photodamage[30,32]. The LPO product was identified in tissue sections by treatment with primary polyclonal rabbit anti-HNE antibodies (1:500, Calbiochem, San Diego, CA, USA) for 18 h at 4 °C. After washing with PBS at RT (3 times – 20 minutes), the bound antibody was detected with secondary goat anti-rabbit Immunoglobulin G antibodies coupled to horseradish peroxidase (HRP) (1:50, Zymed Laboratories, San Francisco, CA) and further developed with 3-amino-9-ethylcarbazole (AEC) (Sigma Chemical Co., St Louis, MO, USA). Samples were taken at the same time points as in histological examination.

Light microscopy was performed using a fluorescent microscope (Nikon Optiphot 2, Tokyo, Japan) equipped with a digital camera (DVC Company, Inc., Austin, TX, USA).

Results

Three weeks after grafting, the tumour was visible as a small lump at the grafting site. The tumours grew approximately 2–7 mm^3 per week (Fig. 1). When tumours reached the size of 6–8 mm in diameter >50 mm^3 (10–12 weeks after grafting), the mice were randomly divided into 6 groups, 8–10 mice each. The average tumour-take in this study was 89% (51/57). In four cases (7.8%), the tumour underwent spontaneous regression, and the mice were sacrificed.

The tumours appeared as a smooth round or oval pale pink friable mass with no signs of lobulation (Fig. 2B). Histologically, the tumours consisted of round large cells with centrally located nuclei, containing smooth to granular chromatin (Fig. 3G). The cytoplasm stained from basophilic to light grey. In addition, clusters of lymphocytic infiltration were observed (Fig. 3G).

In the first part of the study, we examined the response of the bulky XTVT tumour mass to WST09-VTP. Animals with tumours (diameter 6–8 mm) were randomly selected and received one of the three possible treatment protocols: (i) WST09-VTP, (ii) WST09 alone (dark control) or (iii) illumination alone (light control). The experiment included 27 mice. Fifteen mice received VTP. At 24 h post-VTP, the treatment site showed an oedema, which dramatically increased by 48 h. At this time, tumour necrosis at the treatment site was observed in all VTP mice. A large oedematous area developed with gradual crust formation was observed in all

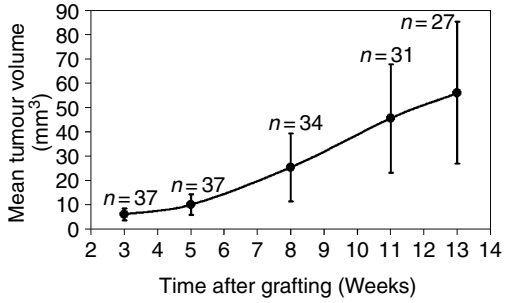

Figure 1. Xenograft-transmissible venereal tumour growth curve: Tumour volume of 37 grafted mice was measured over 13 weeks after implantation. Tumour growth (volume) as function of time is presented. Values are ±SD. The number of mice declined with time when animals were drawn for vascular-targeted photodynamic therapy. All other details were as described in the methods section.

tumours by 6–8 days post-VTP, while the surrounding skin appeared normal. Wound healing was observed beyond 12 days with a complete cure in 13/15 of the cases (86.7%) by 25–35 days after VTP (Fig. 2A). Follow-up of the mice was continued for 90 days post-treatment to ensure no recurrence. Two mice died of an undetermined cause when still at the stage of crust (days 20 and 31 after treatment); one mouse died after wound healing (on day 52 after treatment). Two mice (16.7%) had local tumour regrowth observed by 55 and 83 days post-treatment. Following VTP, the tumour volume declined to undetectable levels, and the observed rate of complete cure (90 days after treatment) was 83.3% (10/12) of the mice. Light ($n = 6$) and

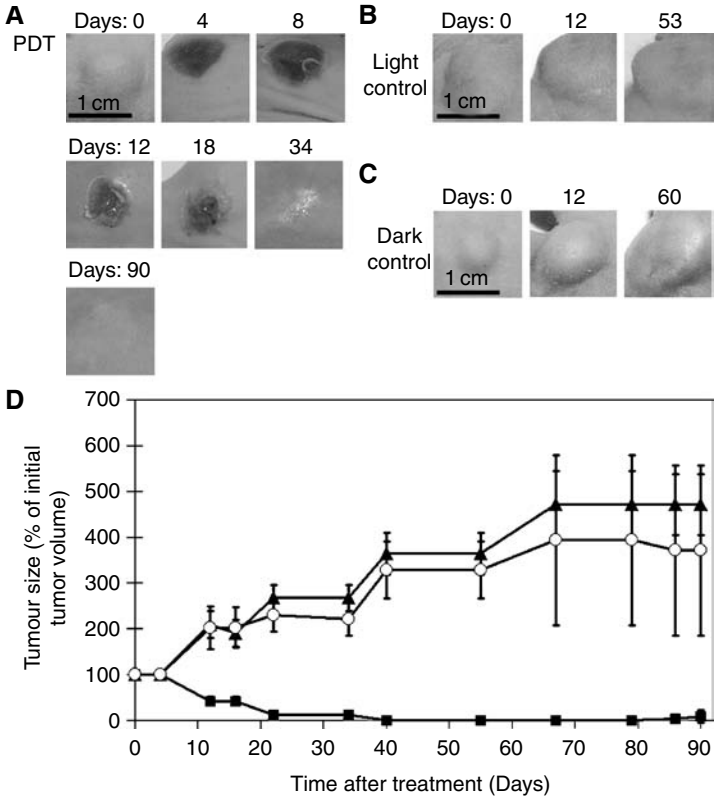

Figure 2. The response of xenograft-transmissible venereal tumour to Pd-bacteriopheophorbide-vascular-targeted photodynamic therapy (WST09-VTP), clinical evaluation: The clinical response to VTP was recorded photographically, and a typical example of three mice representing full treatment and control groups is given. (A) VTP-induced necrosis is observed at 2–12 days post-VTP, followed by healing (with a complete cure and minimal local scaring at 34 days) and no recurrence up to 90 days after VTP with no apparent metastases. In light (B) and dark (C) controls, the tumours continued to grow, and animals were euthanized at 53 (light control) and 60 days (dark control) after treatment due to tumour size >600 mm^3. The tumour volume after treatments (D) was recorded and calculated as described in 'Methods'. Following VTP, a decline in tumour volume was observed (squares, $n = 15$), while in light control (circles, $n = 6$) and dark control (triangles, $n = 6$) tumours continued to grow.

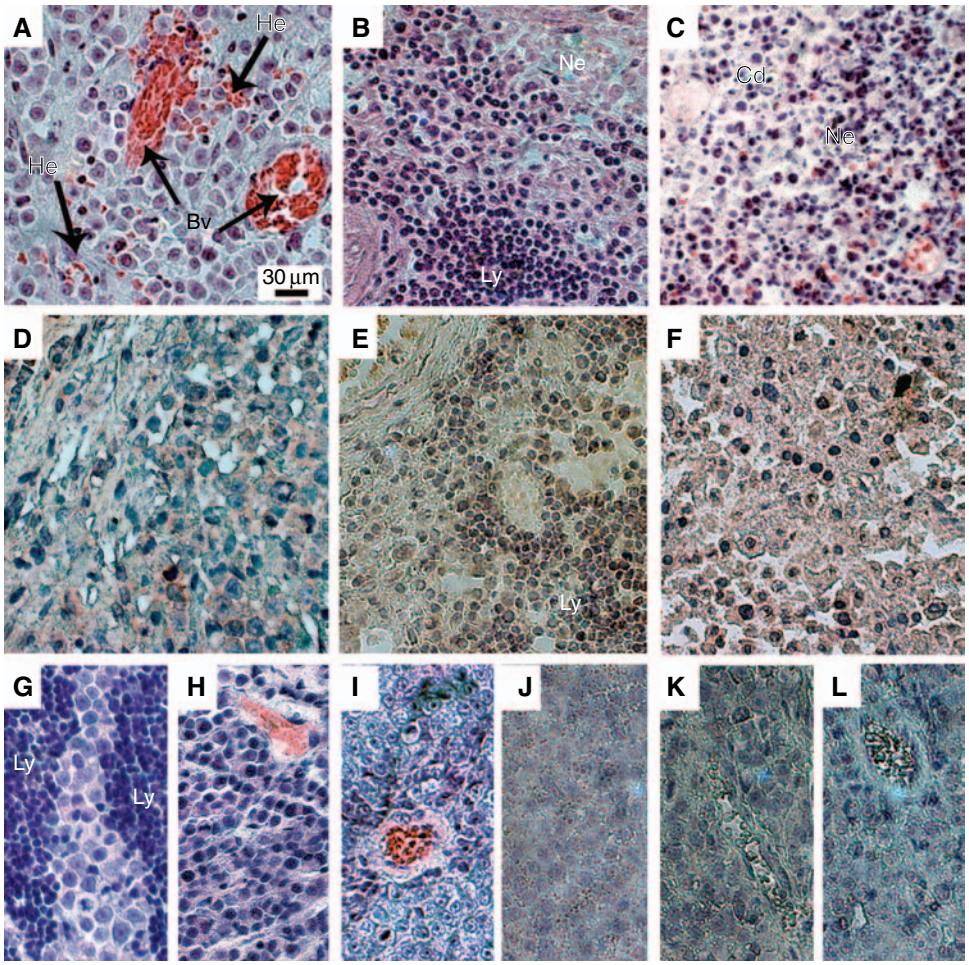

Figure 3. The response of xenograft-transmissible venereal tumour to Pd-bacteriopheophorbide-vascular-targeted photodynamic therapy (WST09-VTP), histopathological evaluation: At 10 h after VTP, (A) congestion of blood vessels (Bv) and multifocal haemorrhage (He) were observed, but tumour cells were viable with some initial necrosis in a few regions. At 24 h (B) and 48 h (C) after VTP, necrosis (Ne) developed, accompanied by lymphocytic (Ly) infiltration. Intact tumour cells are present in the untreated tumour (G), light control (H) and dark control (I). Immunostaining for 4-hydroxy-2-nonenal (HNE) was negative in untreated tumour (J), light (K) and dark (L) controls. Ten hours after VTP, positive HNE staining is observed in some necrotic regions (D). By 24 h (E) and 48 h (F) after VTP, the development of necrosis is accompanied by increased intensive anti-HNE staining of the tumour with negative staining of the Ly infiltrate (E). Bar 30 µm.

dark ($n = 6$) control tumours continued to grow, and the animals were euthanized (CO_2) when the tumour volume reached the maximal allowed volume or at the end of the experiment (90 days after treatment) regardless of tumour size (Fig. 2B,C,D).

Histopathological examination 10 h post-VTP showed haemorrhagic regions around blood vessels, as well as mixed inflammatory infiltration in the surrounding tissues (not shown), while the tumour cells appeared viable, and just a few necrotic regions were seen (Fig. 3A). Degenerative changes in the entire neoplastic cell population with necrosis associated with haemorrhage and lymphocytic infiltration were observed 24 h post-VTP (Fig. 3B). In the surrounding tissue, oedema and mixed inflammatory infiltration were observed. At 48 h after treatment (Fig. 3C), the tumour mass was completely necrotic. The tumour response to WST09-VTP developed from vascular damage early on to complete tumour cell necrosis. Histopathological analysis of the light control

(Fig. 3H) and the dark control tumours (Fig. 3I) revealed intact tumour cells supplied by intact blood vessels. Untreated tumours had the same architecture, except for focal moderate lymphocytic infiltration (Fig. 3G). There was no evidence of spontaneous tumour necrosis in both control groups. At 90 days after VTP, only tumour-free scar tissue could be found at the treatment site.

As a measure for VTP-induced damage, we used local LPO, shown by us previously to result from the VTP-induced oxidative insult during and after the procedure. This injury can be immunohistochemically assessed using HNE as an LPO marker[30,32]. Consistent with photodamage development, HNE staining was first observed in some necrotic regions of the tumour by 10 h after VTP (Fig. 3D). At 24 h post-treatment, the positive HNE staining kept spreading coincidental with necrosis (Fig. 3E), followed by further intensification of HNE staining by 48 h post-VTP (Fig. 3F). In contrast, no staining was observed in the untreated and control specimens (Fig. 3J–L).

Discussion

It is well known that CTVT can be transmitted between dogs and other canids across the major histocompatibility complex. The artificially transmitted tumours usually regress spontaneously after several months, except pups and immunosuppressed dogs[39].

A large majority 51/57 (89%) of the experimental CD1 nude mice used in these experiments developed tumours as soon as 3 weeks after grafting (Fig. 1). Therefore, we can conclude that implanting tumour fragments of 2 × 2 mm size is sufficient to establish a viable TVT model. There was no evidence of metastases, in contrast to the NOD/SCID model[1]; however, in our case, tumour growth was interrupted at a relatively earlier stage. Some spontaneous regression of XTVT was evident (4/51, 7.8%) in agreement with the natural behaviour of transplanted CTVT[39] in dogs.

In comparing the TVT model in CD1 nude mice with the one described in NOD/SCID mice[1], we can find some differences, such as the absence of metastases, spontaneous regression and the failure of tumours to grow in 6/57 (11%) of the mice, which is consistent with the differences between the two mouse strains. The NOD/SCID mice were produced by backcrossing the *scid* mutation onto NOD/Lt background, which is profoundly immunodeficient having multiple defects in innate and adaptive immunologic functions. Thus, NOD/SCID mice are very useful as a model for studying tumours and their metastases, especially the CTVT with its proven immunity[1,40,41].

In this study, we present the application of WST09-based VTP on a CTVT model in the nude mouse. The success of treatment is based on the damage to the tumour vasculature (Fig. 3A) induced by therapeutic intravascular photosensitization as shown earlier[30,32]. In contrast to the appreciable application of PDT for treatment of various indications in humans, the use of this treatment modality in veterinary oncology is still rather limited, with squamous cell carcinoma (SCC) being most frequently reported[42]. In cats, SCC was treated with a phthalocyanine-based PDT protocol with a 59% success rate but with severe side-effects, such as photophobia, swollen faces and skin pigment changes during the first two weeks after treatment[42]. Treatment of SCC with 5-ethylamino-9-diethylaminobenzo[a]phenothiazinium chloride (EtNBS) in dogs and cats showed benefit in 63% of the cases, whereas treatment of ocular melanoma and mast cell tumours were unsuccessful[43]. Chloroaluminium-sulphonated phthalocyanine (CASPc) was used to treat SCC, mast cell tumours and mixed carcinomas in dogs, cats and snakes, with an overall 67% response rate. Skin phototoxicity was not reported, but the photosensitizer was injected 48 h before treatment[44]. PDT has been applied for the treatment of oral SCC in dogs, using photochlor with success rates equal to surgical removal but with better cosmetic results[45]. Photochlor was used to prevent recurrence of haemangiopericytomas in dogs after surgical excision. Infection and delayed wound healing were observed, with no advantage in preventing recurrence[46]. To the best of our knowledge, delayed phototoxicity to photochlor-based PDT was not reported in dogs. Another use of

pyropheophorbide-α-hexyl-ether was reported in treatment of intranasal tumours in dogs and cats. In all cases, there was improvement in clinical signs, without long-term phototoxicity[47]. The use of photochlor in the treatment of early stage SCC in cats gave good results. In 4% (two of 51) of the cats, phototoxicity was noticed within 2 weeks after treatment[48]. In recent studies SnET2[49], a second generation photosensitizer, which allows for deeper light penetration and reduced skin phototoxicity, has been used but with a lower success rate (38% with no recurrence) after the first treatment. However, all recurrent cases responded completely to a second treatment session, so that the reported overall success rate following two treatment sessions was 82%[42]. In most studies, the photosensitizer was administrated 24–48 h before treatment, with some exceptions. PDT with 5-aminolaevulinic acid includes a shorter drug-light time interval (DLTI) of 3–6 h[50,51]. Recently, an even shorter DLTI of 1 h was reported for PDT of dogs and cats in the treatment of superficial carcinoma with photochlor. Interestingly, no cutaneous phototoxicity was noticed in these cases[52].

In the case of WST09-VTP, the significant depth of penetration achieved at the excitation wavelength enables induction of deep necrosis to the depth of 1.3 cm in human prostate tumours[32] and 2 cm in the normal prostate of the dog[29]. This deep light penetration and the practice of interstitial light delivery (allowing tumour ablation to a radius of up to 2 cm around the optic fibre), along with systemic administration of WST09, have become major factors in the early planning and success of VTP in clinical trials of patients with recurrent prostate cancer, following failed external beam radiation[36]. Furthermore, WST09-VTP has been reported to specifically destroy normal prostatic tissue with minimal side-effects, without damaging the dog urethra[29]. It is therefore expected that WST09-VTP will be very useful in treating CTVT with only minimal or no damage to the surrounding tissue. In addition, this protocol may provide the means for treating other tumour types, which have no other effective treatment alternative such as prostatic tumours in dogs[53]. PDT of prostatic carcinoma using 5-aminolaevulinic acid has recently been described with favourable results[54]. It thus seems that PDT could be developed into a useful modality for the treatment of canine prostate cancer. In our lab, the WST09-VTP protocol was developed on well-defined tumours. Single treatment sessions of CTVT were used with a relatively high success rate of 87.6% (using tumour necrosis as the end point, Fig. 2), and a complete cure of 83% by 90 days post-treatment. This cure rate is similar to that obtained with this VTP protocol for treatment of other tumour models (Table 1).

Histopathologically, necrosis was initiated by vascular damage, which led to haemorrhage during the first 48 h after VTP. At this time point, massive destruction of the tumour and complete necrosis were seen (Fig. 3). However, the effect on the surrounding tissue was mild and transient, presumably due to the greater fragility of the tumour neovasculature and the relative resistance of the healthy tissue vasculature.

Table 1. Xenograft models and cure rate with WST09-VTP

Tumour type and animal model	Cure-rate percentage	Reference
XTVT – CD1 nude mice	83	–
Rat C6 glioma – CD1 nude mice	64	Shreiber S *et al.* (2002)
Human small cell carcinoma of the prostate – CD1 nude mice	69	Koudinova NV *et al.* (2003)
Human adenocarcinoma of the prostate – CD1 nude mice	74[a]	Plaks V *et al.* (2004)
Human HT29 colon carcinoma WT/MDR+ – CD1 nude mice	82/88[a]	Preise D *et al.* (2003)
Rat DS sarcoma – Wistar rat	78[b]	Kelleher DK *et al.* (2003)
Rat DS sarcoma – Wistar rat	92	Kelleher DK *et al.* (2004)
Mouse M2R melanoma – CD1 nude mice	80[b]	Zilberstein J *et al.* (2001)

MDR, multidrug-resistant; XTVT, Xenograft-transmissible venereal tumour.
[a]Tumour necrosis as endpoint.
[b]Using bacteriochlorophyll serine as photosensitizer.

It was shown previously that PDT with photofrin in mice led to macromolecular leakage from blood vessels and to vascular constriction during the illumination[55]. Moreover, vascular damage and subsequent tumour cell anoxia caused by photofrin PDT led to tumour necrosis, even though the tumour vasculature was not the primary target of the treatment[56,57]. In contrast, in PDT with bacteriochlorophyll derivatives as sensitizers, vascular insult, macromolecular leakage and blood stasis play a primary role in tumour destruction, while tumour cell death due to ischaemia seems secondary as previously reported[28,32]. The results obtained in the present study with CTVT confirm earlier observations of the considerable efficacy of WST09-VTP in other tumours[30,32,34,35].

One of the suggested cytotoxic mechanisms of photosensitization is based on local ROS formation and LPO[55,56,58]. LPO leads to cell injury and death as described for spontaneous oxidative stress associated with numerous diseases[59]. It was previously shown by this group that VTP-induced oxidative damage can be detected by positive staining for LPO products in treated tumours[30,32,34]. Peroxides of polyunsaturated fatty acids are converted into aldehydes such as HNE, which are highly reactive by themselves and considered secondary toxic agents that disseminate and augment initial free radical events[60,61]. HNE can spontaneously conjugate to proteins and thus be immunohistologically traced with anti-HNE antibodies and serve as an LPO marker[62].

In this study, untreated tumours as well as dark and light controls contained viable tumour cells, which stained negatively for HNE (Fig. 3J–L). Following VTP, the development and spreading of LPO and necrosis were observed. At 10 h after treatment, LPO was observed only in a few scattered regions (Fig. 3D). Further spreading of the LPO process due to free radical chain reactions and diffusion of cytotoxic LPO products was coincident with massive necrosis at 24–48 h post-VTP (Fig. 3F,E). As was shown by us following WST09-VTP of human prostate xenografts, LPO was first detected as soon as 1 h after treatment, in the boundary of the tumour blood vessels before spreading to the surrounding tumour cells[32].

Our study supports previous results which have shown that the primary target of WST09-VTP is the tumour vasculature[32]. The damage process initiated in tumour vasculature spreads as a second wave of light-independent LPO. The second wave is a result of ischaemia, created by blood stasis that spreads through the entire mass of the tumour and leads to total necrosis (Fig. 3)[32].

Although CTVT usually responds well to the chemotherapy and is rarely metastatic, some cases of non-responders have been reported in the literature[13,15–17,63,64]. Our results predict that WST09-based VTP may be useful in treating such cases based on the positive response of drug-resistant HT29 tumours[34]. Moreover, this protocol is rather short, and the best time estimate for such a procedure would be the one recently reported in the treatment of human prostate cancer (20–30 minutes of illumination that partially overlap with 17 minutes of drug infusion[36]. This estimate excludes the preparatory steps and anaesthesia. As such, this protocol would fit conveniently to the ambulatory clinic. It should also be considered that the treatment protocol with WST09 can be performed in a single visit, whereas treatment with most other PDT drugs requires two visits (one for photosensitizer administration and one for illumination). The rapid clearance of the drug from the circulation (minutes) and from the body (24–48 h in mice, rats and humans) minimizes cutaneous phototoxicity[28]. It should be noticed that WST09 is presently administrated with Cremophor EL as vehicle. The most common side-effect of Cremophor EL in humans and dogs is a hypersensitivity reaction[65]. Therefore, H2-receptor blockers and corticosteroids are administrated along with slow-rate drug infusion to prevent this reaction[29]. This protective treatment may become unnecessary in the future when new vehicles may be introduced. Due to relatively inexpensive equipment and minimally invasive treatment protocol, this methodology can be easily adopted for common veterinary practice. Therefore, WST09-based VTP seems to be a promising mode of treatment for CTVT and might be

considered for treatment of other tumour types in veterinary and human medicine.

Acknowledgments

Y. Salomon is the incumbent of the Tillie and Charles Lubin Professorial Chair in Biochemical Endocrinology. A. Scherz is the incumbent of the Robert and Yaddele Sklare Professorial Chair in Biochemistry. The authors wish to thank Mrs Ester Shai for her helpful assistance and Dr Ori Brenner for help in the pathological analysis. We thank Ms Rina Tzoref for her devoted secretarial assistance. This study was supported by Steba Biotech and Negma Lerads, France.

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