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The *in vivo* efficacy of phthalocyanine–nanoparticle conjugates for the photodynamic therapy of amelanotic melanoma

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ABSTRACT

The efficiency of a Zn(II)-phthalocyanine disulphide (C11Pc), a compound with both phthalocyanine units bearing seven hexyl chains and a sulphur terminated C11 chain, as a photodynamic therapy (PDT) agent was investigated in C57 mice bearing a sub-cutaneously transplanted amelanotic melanoma. The phthalocyanine was intravenously injected at a dose of 1.5 μmol/kg body weight either free or bound to gold nanoparticles, using a Cremophor emulsion as a delivery vehicle. Biodistribution studies at selected post-injection times showed that the nanoparticle-associated C11Pc was recovered in significantly larger amounts from all the examined tissues and the serum and yielded a greater selectivity of tumour targeting: thus, the ratio between the amount of phthalocyanine recovered from the amelanotic melanoma and the skin (peritumoural tissue) increased from 2.3 to 5.5 from the free to the gold nanoparticle-bound C11Pc at 24 h after injection. PDT studies with the C11Pc-loaded amelanotic melanoma showed a markedly more significant response of the tumour in the mice that had received the nanoparticle-bound photosensitiser; the PDT effect was especially extensive if the irradiation was performed at 3 h after C11Pc injection when large phthalocyanine amounts were still present in the serum. This suggests that the PDT promoted by C11Pc predominantly acts via vascular damage at least in this specific animal model. This hypothesis was fully confirmed by electron microscopy observations of tumour specimens obtained at different times after the end of PDT, showing an extensive damage of the blood capillaries and the endothelial cells.

1. Introduction

Photodynamic therapy (PDT) is a well-established modality for the treatment of localised tumours which is used in clinical practice as an alternative or adjuvant to conventional therapies, such as radiotherapy, surgery or chemotherapy. PDT selectively destroys neoplastic lesions by the combined action of a light-activated drug, termed a photosensitiser, and visible light. The wavelengths of the light that are typically used for PDT are in the red or near infrared spectral range as these wavelengths exhibit a greater penetration depth into most human tissues and are not absorbed by normal tissue constituents. Upon irradiation, the photosensitiser is promoted to the long-lived lowest excited triplet state. The energy of the excited state photosensitiser molecule is transferred to the ground state of oxygen to produce the singlet

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state oxygen species. Singlet oxygen is cytotoxic and it is the production of this species which results in the destruction of cancerous tissue.[1] Alternatively, the triplet photosensitiser can promote electron transfer processes with nearby substrates with the generation of radical species (including the superoxide anion and the hydroxyl radical), which can in turn induce different types of damage in the microenvironment of the photosensitiser binding site.[1]

Approval of PDT for oncological indications was first granted in 1993. Currently four drugs have received regulatory approval in North America and/or the European Union[2]: Photofrin (a complex mixture of haematoporphyrin derivatives, Axcan Pharma Inc., Mont-Saint-Hilaire, Canada), for advanced and early lung cancer, superficial gastric cancer, oesophageal adenocarcinoma, cervical cancer and bladder cancer; Foscan (meta-tetrahydroxyphenyl chlorin, Biolitec Pharma Ltd., Dublin, Ireland), for palliative head and neck cancer; the other two drugs, Levulan (5-aminolevulinic acid; ALA, Dusa Pharmaceuticals Inc., Wilmington, MA, USA) and Metvix (methyl 5-aminolevulinate, Photocure ASA, Oslo, Norway), are not themselves photoactive but when applied to basal cell carcinoma, for example, are metabolically converted to protoporphyrin IX (or a methyl ester derivative for Metvix) *via* the haem biosynthetic pathway. Protoporphyrin IX is a photosensitiser and consequently produces singlet oxygen when stimulated by light. Photodynamic therapy with these four photosensitiser drugs has been used to treat several thousand patients worldwide.[3,4] While excellent clinical results are achieved, the full exploitation of the potential of PDT is impeded by factors such as the somewhat limited selectivity of tumour targeting; the prolonged persistence of the available PDT agents in the skin, causing a generalised cutaneous photosensitivity; and the chemical heterogeneity and inefficient red light absorption typical of Photofrin and ALA-derived protoporphyrin.[1,3] The clinical status of photodynamic therapy for cancer treatment has been recently reviewed.[2–4]

Novel approaches are being investigated in order to overcome the present limitations of PDT, including the development of second-generation photosensitisers with improved photochemical and tumour-localising properties. Many of the compounds developed as second-generation photosensitisers have limited water solubility as hydrophobic photosensitisers typically exhibit a better tumour targeting and substantially greater PDT efficacy.[1,5] The obvious limitation of such water-insoluble photosensitisers is that they require a vehicle to deliver the drug to the tumour tissue.

While numerous drug delivery vehicles exist, particularly encouraging results have been obtained recently which suggest that nanoparticle-carried PDT agents are accumulated in significant amounts by a variety of tumour cells.[6–9] Upon accumulation in the tumour and subsequent activation by suitable visible-light wavelengths, these photosensitiser–nanoparticle conjugates induce an efficient damage of the malignant tissue.[9–11] Photophysical investigations have demonstrated that the association of a photosensitiser with nanoparticles does not necessarily alter its excited state properties nor the efficiency of singlet oxygen generation.[10–12] Thus, PDT could take advantage of the increasing evidence supporting the concept that nanoparticles have a large potential to act as a viable enhancer of tumour targeting by anti-neoplastic drugs.[13,14]

Many different materials have been used to formulate nanoparticles for drug delivery. Various photosensitisers, many of which are hydrophobic, have been encapsulated within water-soluble polymers such as polylactic-co-glycolic acid and polylactic acid, to overcome the problems associated with solubilisation. For example, *meso*-tetra(4-hydroxyphenyl)porphyrin,[15,16] bacteriochlorophyll-a,[17] verteporfin,[18] methylene blue,[19] hypericin[20] and various phthalocyanines[21–23] have all been encapsulated in such water-soluble polymers. Silica nanoparticles have been used successfully for the delivery of the readily available PDT photosensitisers, such as pheophorbides and chlorins,[7,24] or with unusual sensitisers, such as fullerene.[25] Cyclodextrin-based nanoparticles also have been used to entrap photosensitisers and shown to exhibit good PDT efficiency.[26,27]

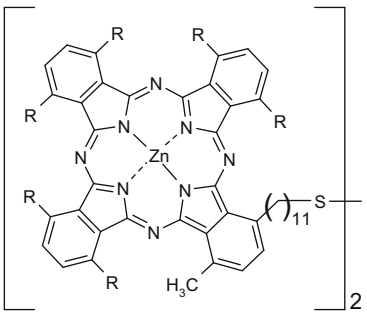
We[28,29] and others[30] have developed gold nanoparticles as carriers of hydrophobic phthalocyanines, examples of second-generation photosensitisers. In this instance, rather than the photosensitiser being encapsulated within the nanoparticle carrier, the photosensitiser is bound to the surface of the gold nanoparticles via a thiol tether specifically designed so that the molecule will form a self-assembled monolayer on the gold nanoparticle surface.[28] The formation of the phthalocyanine on the gold nanoparticle surface has the potential advantage that the hydrophobic character of the photosensitiser is maintained so that the conjugates retain the enhanced tumour targeting and greater PDT efficacy. It has also been suggested that the binding of the photosensitiser to the surface of the nanoparticle may represent an advantage as singlet oxygen does not need to diffuse out of the polymeric/silica nanoparticle structure as per encapsulated photosensitisers.[11]

While the *in vitro* results are extremely promising, to date there are only a limited number of studies in which the *in vivo* efficacy of photosensitiser–nanoparticle conjugates has been investigated.[18,30–35] In this paper, we describe the pharmacokinetic behaviour and PDT efficacy of phthalocyanine–gold nanoparticle conjugates for the treatment of a sub-cutaneously implanted amelanotic melanoma. This tumour was selected as a model for the present investigations since it is a common skin tumour that favourably responds to PDT treatment[36] and it has been repeatedly adopted for assessing the efficacy of the experimental PDT with a number of photosensitising agents.[37,38]

2. Materials and methods

2.1. *Photosensitising agents*

The following photosensitising agents were used in the pharmacokinetic and experimental PDT studies: 1,1′,4,4′,8,8′,15,15′,18,18′,22,22′-tetradecakisdecyl-25,25′-(11,11′dithiodiundecyl)diphthalocyanine (C11Pc), (the free, non-bound, phthalocyanine photosensitiser) and the nanoconstruct made by conjugating the C11Pc photosensitiser with gold nanoparticles (C11Pc-Np). The chemical structure of the C11Pc is shown in Fig. 1. The synthesis of C11Pc and the formulation of the C11Pc-Np nanoparticle conjugate have been previously



R= *n*-C_6H_{13}

Fig. 1 – Chemical structure of the C11Pc phthalocyanine photosensitiser used in this study.

reported.[28,29] With their hydrophobic nature, both the C11Pc and the C11Pc-Np conjugate samples were dispersed in a Cremophor EL emulsion before systemic administration to the animals. The incorporation of these photosensitisers into the oil emulsion was performed according to a previously described procedure.[37] The amount of phthalocyanine embedded within the Cremophor was determined by dissolving a known volume of the emulsion into a known excess of tetrahydrofuran and measuring the absorption spectrum. The phthalocyanine concentration was obtained by using a molar extinction coefficient value of 223,000 M^{-1} cm^{-1} at 696 nm for both the free and the nanoparticle-bound C11Pc (the two samples exhibited identical absorption spectra in the red spectral range).

2.2. *Cell line and culture conditions*

Cells from the B78H1 cell line, an amelanotic clone of murine melanoma, were initially provided by Istituto di Cancerologia (Bologna) and originally obtained from B16 melanoma. The cells were cultured as a monolayer at 37 °C in a dark humidified atmosphere with 5% CO_2 and were grown in Dulbecco's modified minimal essential medium (D-MEM, Sigma Chemical Co., St. Louis, USA) containing 10% heat-inactivated foetal calf serum (FCS, Gibco Invitrogen, Como, Italy) and were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin.

2.3. *Pharmacokinetic studies*

Female C57/BL6 mice (18–20 g body weight) obtained from Charles River (Como, Italy) were used as experimental models. The B78H1 amelanotic melanoma cells were transplanted into the upper flank of the mouse by subcutaneous injection of 20 μl (10^6 cells) of a sterile cell suspension in phosphate-buffered saline (PBS).

At about 3 weeks after tumour transplantation the mice (at least five mice per group) were injected into the tail vein with the selected photosensitiser formulation. In particular, 1.5 μmol of phthalocyanine per kg body weight was administered in the case of both the C11Pc and C11Pc-Np derivatives; the dose of i.v. injected photosensitising agent was limited by the need to avoid the onset of anaphylactic reactions in the mice as a consequence of the direct introduction of too large an amount of Cremophor into the bloodstream.[38]

In all cases, the mice were kept in standard cages with free access to tap water and normal dietary chow. At predetermined times after intravenous injection of the photosensitising agent, groups of five mice were sacrificed by euthanasia: blood samples were rapidly taken and centrifuged at 3000 rpm for 10 min at room temperature using plastic test tubes in order to remove the blood cells. The sera thus collected were pooled and 50-fold diluted with 2% aqueous sodium dodecyl sulphate (SDS, a product of Prolabo, Fontenay-sous-Bois, France). At the same time, the tumour and selected normal tissues were rapidly excised, washed with physiological solution and a known amount of tissue (ca. 200 mg) was homogenised in 2% aqueous SDS (2 ml) using a Potter vessel; the homogenate was incubated for 1 h at room temperature under gentle magnetic stirring, then 0.25 ml of the suspension was diluted with tetrahydrofuran (2.25 ml) and centrifuged at 3000 rpm for 10 min. Both the serum and the tissue extracts were assayed for the C11Pc content by measurement of the 640 nm-excited phthalocyanine fluorescence emission in the 660–850 nm spectral interval. The fluorescence intensity was converted into C11Pc concentration (expressed as nanomoles of photosensitiser per g of tissue) by interpolation with a calibration plot. Previous investigations[39] showed that this procedure yields a >90% recovery of the phthalocyanine from the tissues.

In a parallel set of studies, healthy mice intravenously injected with the C11Pc-Np (3.0 μmol/kg body weight) were kept in metabolic cages and both the urine and faeces were collected for 1 week and analysed for the phthalocyanine content at 24 h intervals.

2.4. *Photodynamic therapy studies*

At 10–15 d after subcutaneous injection of the B78H1 cells, when the tumour external diameter was in the 0.4–0.6 cm range, the C11Pc or the C11Pc-Np samples in a Cremophor emulsion were injected into the mouse caudal vein (at least eight mice per experimental group) at a dose of 1.5 μmol/kg body weight.

Irradiation of the amelanotic melanoma in tumour-bearing mice was performed at two time intervals (3 and 24 h) after i.v. injection of the photosensitising agent by using the 600–700 nm wavelength range, which was isolated by a set of bandpass filters from the emission of a quartz-halogen lamp (Teclas, Lugano, Switzerland). The light source was operated at a fluence-rate of 175 mW/cm^2 for a total fluence of 157 J/cm^2.

The effectiveness of the treatment was evaluated by comparing the rate of tumour growth as a function of the post-irradiation time for the photosensitised mice with that observed for control mice that had not been exposed to light and had not been injected with the photosensitiser. The tumour size was measured at daily intervals by means of a calliper. Individual tumour volumes (V) were calculated assuming a hemiellipsoidal structure for the tumour nodule and measuring the two perpendicular axes (a and b) and the height (c). Application of the relationship $V = 2/3\pi$ $(a/2 \times b/2 \times c)$ yielded the tumour volume. Mice were followed daily

to determine the day on which the tumour volume equalled or exceeded 400 mm^3 (time to 400 mm^3): at this time, mice were sacrificed by euthanasia in line with the rules established by the University of Padova ethical committee for humane treatment of experimental animals. In no instance was a spontaneous regression of the transplanted tumour observed.

2.5. *Electron microscopy studies*

The tumour-bearing mice were intravenously injected with either C11Pc or C11Pc-Np (1.5 μmol/kg) and at 3 h after injection the neoplastic lesion was exposed to 175 mW/cm^2 light (600–700 nm) for 15 min (157 J/cm^2). At predetermined times (3, 6 and 24 h) after the end of PDT, three mice per group were sacrificed and the tumour was quickly removed. Specimens of macroscopically non-necrotised tumour tissue were taken, fixed in 3% glutaraldehyde, cacodylate-buffered at pH 7.3, for 2 h at 4 °C, post-fixed in 1% OsO_4 cacodylate-buffered for 1 h, dehydrated and embedded in Epon. The following control groups were also examined by the procedure described above: (i) mice injected with the photosensitiser and not exposed to light and (ii) untreated and unirradiated mice. Each group consisted of three mice.

Semi-thin sections of the tumour tissue were then analysed by light microscopy. A significant area was chosen and ultrathin sections (80 nm) were cut, counterstained with uranyl acetate and lead citrate and finally analysed by a Hitachi H-600 transmission electron microscope (TEM).

2.6. *Statistical analysis*

All the pharmacokinetic and photoinactivation data are reported as mean ± standard deviation (s.d.).

3. Results

3.1. *Pharmacokinetic studies with C11Pc and the C11Pc-Np conjugate*

The time-dependent distribution of the i.v.-injected C11Pc in C57 mice bearing a sub-cutaneously transplanted amelanotic melanoma (Fig. 2A) is similar to that previously determined for structurally analogous octa-decyl or octa-pentyl phthalocyanine derivatives albeit in a different animal model.[40,41] Thus, the largest accumulation of the photosensitising agent was found to occur in the constituents of the reticulo-endothelial system, such as liver and spleen. This observation is in agreement with the known tendency of hydrophobic PDT agents to be eliminated from the organism via the bile-gut pathway.[42] The concentration of the phthalocyanine that was recovered from the liver and spleen peaked at 24 h after injection and appeared to gradually decrease up to at least 168 h. Low amounts of C11Pc (<2.5 nmoles/g) were found in a number of other tissues, including brain, kidney and lung (Fig. 2A) at all the post-injection times analysed. Such low concentrations of photosensitiser minimise the risk of undesired side-effects arising from photosensitivity effects at the level of these anatomical sites. The main difference between the pharmacokinetic behaviour of C11Pc as compared with

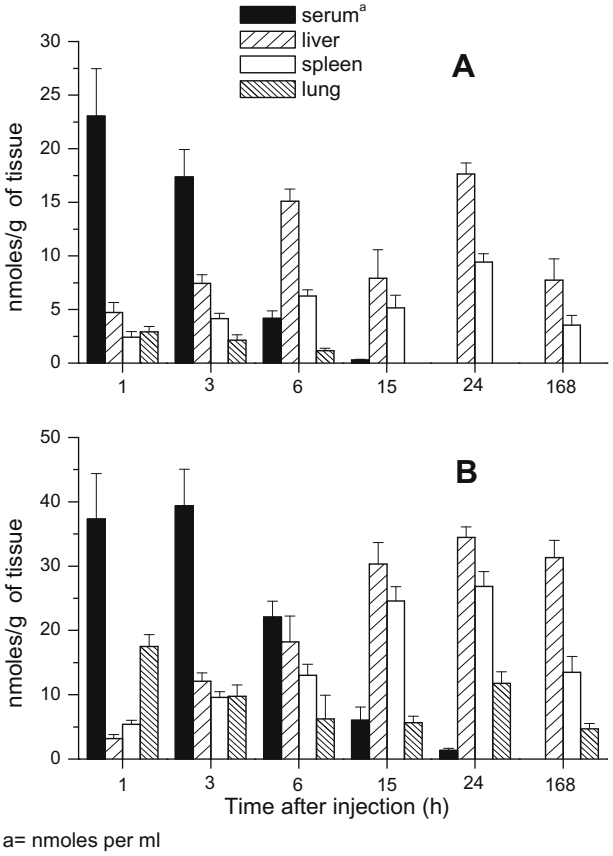


Fig. 2 – Recovery of C11Pc from serum and selected tissues of C57BL/6 mice bearing a sub-cutaneously transplanted amelanotic melanoma. The mice were i.v. injected with 1.5 μmol/kg C11Pc (A) or C11Pc-Np (B) solubilised in a Cremophor EL emulsion.

previously studied phthalocyanine derivatives[40,41] is represented by its relatively fast clearance from the serum, the examination of which at 15 h after injection showed the presence of only residual traces of C11Pc (Fig. 2A). Whether this difference in behaviour is due to the different mouse strain used (C57BL/6 versus Balb/c mice) has yet to be established.

A significantly different scenario was found to occur when the pharmacokinetics of the C11Pc-Np sample was studied. As shown in Fig. 2B, markedly larger phthalocyanine recoveries from the serum, as well as from liver and spleen, were measured, even though identical doses of C11Pc had been injected. Significant amounts of the C11Pc photosensitiser were also found in the lung, especially at short post-injection times, probably owing to the possible capture of colloidal particles by lung capillaries.[43] Moreover, a longer persistence of the nanoparticle-associated C11Pc in all the organs was observed: thus, the phthalocyanine concentration in the liver was essentially constant in the 30–35 nmoles/g range between 15 and 168 h after injection; similarly, the C11Pc concentration in the serum, initially reaching a level of about 37 nmoles/ml, began to decrease only after 3 h from injection, while phthalocyanine amounts close to the lowest detection limit of our analytical method (ca. 0.8 nmoles/ml) were present after 24 h. Once again, only traces of C11Pc were recovered from brain and kidney, indicating that the gold

nanoparticle-conjugated C11Pc (a) cannot cross the blood–brain barrier and therefore should not cause possible neurological damage, and (b) should undergo no significant clearance in the urine. This was experimentally confirmed by analysis of the faeces and urine collected from mice that had been i.v. injected with the nanoparticle-bound C11Pc and kept in metabolic cages for about 1 week after administration of the photosensitising agent: significant amounts of the phthalocyanine (ca. 40 nmoles/g) were recovered in the faeces during the initial 48 h after administration, while no traces of the photosensitising agent were found in the urine throughout the 7 d observation period.

Most importantly, the delivery of the C11Pc using the gold nanoparticle vehicle resulted in a larger photosensitiser accumulation in the tumour compared with that of the free C11Pc at post-injection times longer than 15 h (see Table 1). Moreover, the nanoparticle–associated phthalocyanine displayed a significantly greater selectivity of tumour targeting as expressed by the ratio between the recovery from the tumour and the peritumoural cutaneous districts: thus, the tumour to skin selectivity ratio increased (from the free C11Pc photosensitiser to the nanoparticle conjugates) from 2.6 to 3.7 at 3 h, and from 2.3 to 5.5 at 24 h (Table 1). Such an increase in the ratio essentially reflects a larger uptake and/or retention of the phthalocyanine in the neoplastic tissue since the recoveries of the photosensitising agent from the skin are almost identical for the mice that had been injected with C11Pc or C11Pc-Np.

In summary, the administration of C11Pc via gold nanoparticles results in

- a longer persistence in the blood circulation (serum half-life is 3.5 h and 6 h for the free and conjugated phthalocyanine);
- a larger accumulation of the phthalocyanine in most organs at post-injection times longer than 15 h: this is particularly important for the amelanotic melanoma which undergoes a two- to three-fold increase in the phthalocyanine concentration; similar observations were obtained for the serum, which could favour a PDT action through vascular damage of the neoplastic lesion (see below);
- a prolonged persistence of the photosensitising agent in the liver with no apparent decrease in the phthalocyanine concentration up to at least 1 week; the implications of this finding are examined in Section 4.

3.2. *PDT studies with the C11Pc and the C11Pc-Np conjugate*

On the basis of the data obtained from the pharmacokinetic investigations, PDT studies with C11Pc and C11Pc-Np were performed at two post-injection times, namely 24 h (corresponding to the largest accumulation of the photosensitising agent in the neoplastic lesion) and 3 h (when significant amounts of phthalocyanine are still present in the serum). The tumour response to the PDT treatment was evaluated by following the rate of tumour growth as a function of the post-irradiation time.

As shown in Fig. 3, the amelanotic melanoma grows quite aggressively in control untreated mice, which would eventually lead to death of the tumour-bearing animals after about 3 weeks. However, in our experiments, the mice were sacrificed by euthanasia when the tumour volume reached 0.8 cm^3, according to the rules recommended by the University of Padova ethical committee to minimise unnecessary suffering of the animals. Preliminary studies suggested that no significant difference in the rate of tumour growth was caused by irradiation of the mice in the absence of photosensitiser using the same irradiation regime adopted for the PDT experiments with the phthalocyanine-injected animals. Analogously, injection of C11Pc or C11Pc-Np to the tumour-bearing mice without subsequent red light irradiation had no detectable effect on the growth rate of the amelanotic melanoma.

A barely detectable delay in the rate of tumour growth was caused by PDT treatment at 24 h after injection of both C11Pc and C11Pc-Np, since the plots describing the time-dependent increase in the volume of the amelanotic melanoma for these groups of mice essentially overlapped that observed for control animals except during the 2 d immediately following the exposure to light. However, the tumour response became clearly significant when the irradiation protocol was performed at 3 h after injection of both the free and nanoparticle-conjugated phthalocyanine, notwithstanding the smaller overall amount of photosensitiser which was present in the neoplastic lesion at this time interval. Thus, both groups of photosensitised mice ($n = 10$) remained tumour free for about

Table 1 – Recovery of C11Pc from tumour and skin (peritumoural tissue) at selected times after i.v injection of 1.5 μmol/kg of free or gold nanoparticle-bound phthalocyanine to C57 mice bearing a sub-cutaneously transplanted B78H1 amelanotic melanoma. The recoveries are expressed as nmoles of phthalocyanine per mg of tissue.

Time after injection (h)	C11Pc		C11Pc-Np	
	Tumour	Skin	Tumour	Skin
1	0.81 ± 0.37	0.36 ± 0.05	Traces	Traces
3	0.52 ± 0.21	0.20 ± 0.09	0.44 ± 0.10	0.12 ± 0.06
6	0.64 ± 0.08	0.22 ± 0.05	0.64 ± 0.11	0.32 ± 0.11
15	0.43 ± 0.13	0.23 ± 0.12	1.18 ± 0.36	0.21 ± 0.17
24	0.62 ± 0.25	0.27 ± 0.17	1.16 ± 0.43	0.21 ± 0.12
168	Traces	Traces	0.15 ± 0.07	0.22 ± 0.07

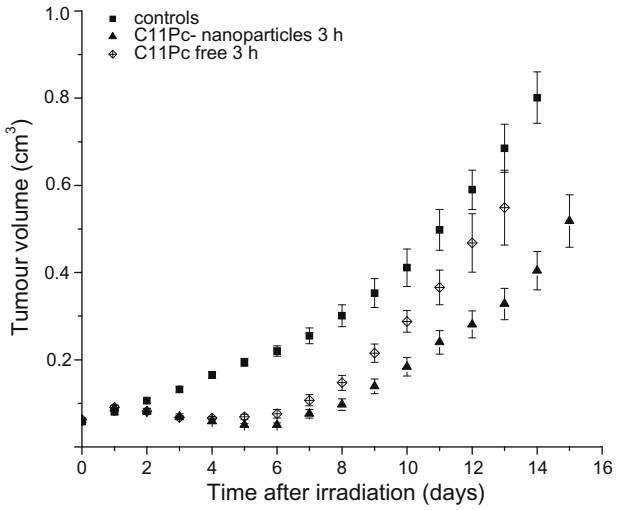


Fig. 3 – Tumour growth rate in C57BL/6 mice bearing a subcutaneously transplanted amelanotic melanoma. The tumour lesion was subjected to PDT treatment (600–700 nm, 175 mW/cm^2, and 157 J/cm^2) at 3 h after i.v. injection of 1.5 μmol/kg C11Pc or C11Pc-Np solubilised in a Cremophor EL emulsion.

6 d after the end of the phototreatment. However, the subsequent tumour growth, which involved all the animals, was appreciably faster for the mice which had received the free C11Pc photosensitiser. Taking a tumour volume of 0.4 cm^3 as a reference point, one can calculate from the plots shown in Fig. 3 a delay of 2 and 5 d for the mice subjected to PDT at 3 h after injection of the C11Pc and the C11Pc-Np samples, respectively.

Information on the mechanisms involved in the tumour photodamage induced by both the C11Pc and C11Pc-Np upon PDT treatment at 3 h post-injection was provided by electron microscopy analysis of tumour specimens obtained at selected post-irradiation times (Fig. 4). Tissues from three independently treated mice were analysed at each time point; in general, good agreement was found for the data obtained with different specimens from each animal.

Typically, the tumours from control C57BL/6 mice (Fig. 4A) were composed of densely arranged cells of polygonal or slightly elongated shape. The nucleus was often rather large with a prominent nucleolus, while the cytoplasm was reduced with few profiles of rough endoplasmic reticulum and scattered mitochondria. The capillaries supplying blood to the neoplastic cells appeared to be of a continuous type and were characterised by a thin endothelium. The ultrastructural features of tumours isolated from mice that received either the C11Pc or C11Pc-Np samples and were not exposed to light (Fig. 4B) were essentially identical with those observed for the untreated mice: this suggests that neither the phthalocyanine nor the gold nanoparticle vehicle induced any detectable alteration in the morphological properties of the amelanotic melanoma tumour. Analogously, no appreciable ultrastructural modification was observed for tumour specimens obtained from mice exposed to light without previous injection of either of the photosensitising agents.

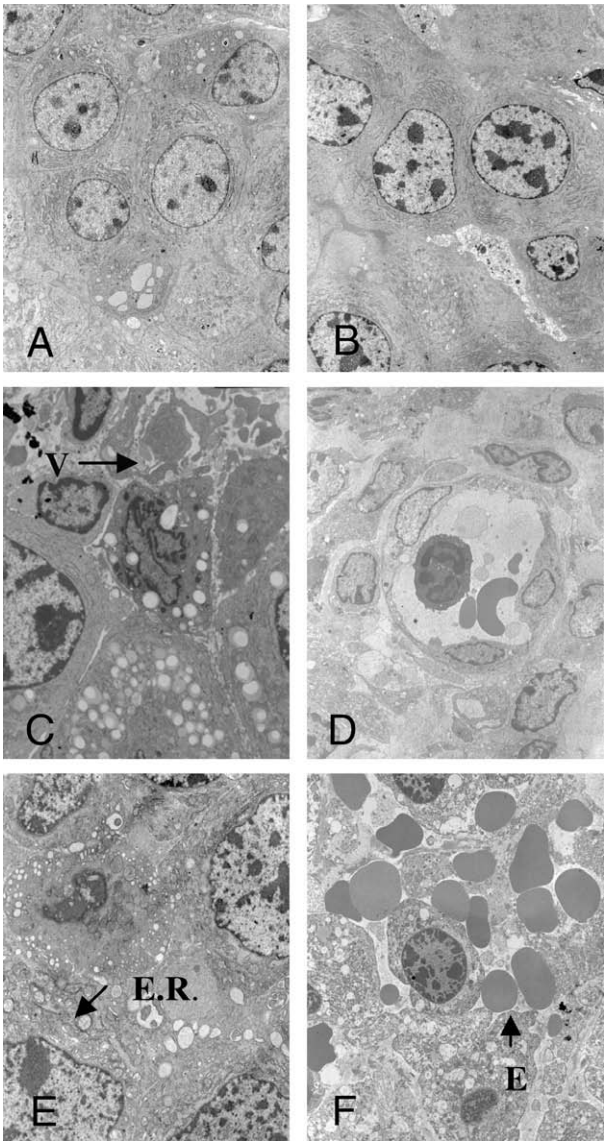


Fig. 4 – Typical micrographs of tumour specimens (amelanotic melanoma B78H1) obtained from untreated and PDT-treated mice. (A) Control untreated mice (3000×), (B) mice injected with 1.5 μmol/kg C11Pc-Np (3000×), (C) mice at 6 h after PDT treatment in the presence of C11Pc (5000×) V altered blood vessel, (D) mice at 24 h after PDT treatment in the presence of C11Pc (3000×), (E) mice at 6 h after PDT treatment in the presence of C11Pc-Np (5000×) E.R endoplasmic reticulum, and (F) mice at 24 h after PDT treatment in the presence of C11Pc-Np (3000×) E erythrocytes. In all cases, the mice were i.v. injected with 1.5 μmol/kg phthalocyanine and PDT treatment involved irradiation with 600–700 nm light, 175 mW/cm^2, and 157 J/cm^2 at 3 h after injection.

At 6 h after PDT in the presence of the C11Pc photosensitiser (Fig. 4C), only minor alterations of neoplastic cells were found to occur, including some swelling of the mitochondria; on the other hand, a number of endothelial cells exhibited an initial damage within 6 h after PDT, as shown by the

appearance of a non-continuous endothelium. Thus, the vascular damage appears to occur at a faster rate and to a greater extent than the damage of neoplastic cells. This difference is more evident in the micrographs taken at 24 h after PDT (see a typical example in Fig. 4D), where a more pronounced damage of the capillaries is observed. These features were markedly more evident in the case of tumour specimens obtained from mice injected with the C11Pc-Np conjugates (Fig. 4E and F), especially at 24 h after PDT: the neoplastic tissue is of haemorrhagic type with a large number of erythrocytes which have clearly leaked out of the capillaries as a consequence of heavy endothelial damage; moreover, an extensive vacuolisation is induced with simultaneous alterations of selected cell organelles, e.g. the endoplasmic reticulum labelled in Fig. 4F.

4. Discussion

The utilisation of delivery vehicles for the administration of photodynamic agents by systemic routes has been the subject of active debate and several experimental studies among both basic investigators and clinical users in the field of PDT.[43] In practice, the photosensitisers which usually exhibit the largest affinity for tumour tissues are characterised by a relatively high degree of hydrophobicity, hence their direct intravenous injection into the bloodstream is greatly facilitated by their previous incorporation into lipophilic carriers, including liposomes, oil emulsions, inclusion complexes and serum proteins.[1,40] The use of such carriers allows the injection of larger amounts of photosensitiser overcoming the problem of the low water solubility and often reduces the extent of photosensitiser aggregation which is favoured by polar media and is known to decrease the efficiency of the overall photodynamic process.[41] Thus, two of the PDT agents which are presently involved in clinical trials, such as Foscan[44] and benzoporphyrin derivative[45] are formulated in association with suitable delivery systems.

The advent of nanoparticles decorated with photodynamically active compounds has unlocked new opportunities for increasing the efficiency and potential of PDT. Thus, nanoparticles having suitable size and geometry can exploit the enhanced vascular permeability and retention effect typical of tumours,[46] especially taking advantage of the more frequent fenestration exhibited by blood capillaries in several malignant tissues.[47] Moreover, nanoparticles can be functionalised with tumour-targeting agents, including antibodies or peptides thereby leading to a more selective loading of the neoplastic cells by the photosensitiser.[14,15,48,49] In this connection, a number of papers published in recent years report the use of nanoparticle-delivered PDT agents with objectively encouraging findings.[13,18,34]

In the present paper, the results of a comparative study of the pharmacokinetic and phototherapeutic properties of a free (C11Pc) and gold nanoparticle-bound (C11Pc-Np) phthalocyanine in a murine tumour model are reported. The C11Pc-Np construct had been previously tested in cultured malignant cells and shown to be characterised by a significantly greater yield of singlet oxygen generation and a larger efficiency of photosensitised cell killing as compared with the free C11Pc photosensitiser.[28,29] Our *in vivo* results are in excellent agreement with those obtained with cell cultures: as shown in Fig. 3, the PDT performed at 3 h after injection of the nanoparticle-bound phthalocyanine causes a tumour response which is markedly more extensive than that observed for PDT in the presence of the free phthalocyanine. The delivery via nanoparticles also leads to a ca. twofold larger concentration of the C11Pc photosensitiser in the amelanotic melanoma lesion, although only at post-injection times longer than 15 h (see Fig. 2). A comparative analysis of the biodistribution and phototherapeutic data provides useful information with regard to the mechanism of the C11Pc-photosensitised damage of the subcutaneous tumour.

Thus, a significant delay in tumour growth is obtained if the irradiation is carried out when an appreciable amount of the phthalocyanine is present in the serum. As a consequence, it appears that the partitioning of the photosensitiser in the various districts of the tumour tissue is more important than the total amount of accumulated photosensitiser in determining the efficacy of the PDT treatment, at least in the tumour animal model used in the present investigations. It appears reasonable to hypothesise that the C11Pc predominantly acts via vascular damage, as unequivocally confirmed by the electron microscopy images which show an early and extensive damage of the endothelial cells (Fig. 4). The higher efficiency displayed by the nanoparticle-delivered C11Pc can thus be explained on the basis of the 2.5-fold larger concentration of this conjugated phthalocyanine in the serum at 3 h post-injection as compared with the free phthalocyanine. The anti-angiogenic action promoted by C11Pc-Np-based PDT is in line with various recently proposed approaches, which aim at inhibiting the tumour growth by stopping the blood supply.[50] On the other hand, the poor phototherapeutic activity found for both C11Pc and especially C11Pc-Np at 24 h post-injection, in spite of the much larger overall concentration in the tumour, cannot be explained at the present stage of our investigations. Possible clues for the interpretation of this issue could be provided by studies on the distribution of the phthalocyanine at a subtissular level, which are presently in progress in our laboratory.

The largest recoveries of phthalocyanine for both formulations occur in the liver and spleen. This is likely to reflect the opsonisation or removal of the Cremophor-based emulsions used for the administration of the C11Pc samples by the mononuclear phagocytic system,[51] a process which has also been proposed for unprotected nanoparticles.[52] Such a sequestration would decrease the average serum lifetime of the photosensitiser which represents a critical factor for the success of PDT, as discussed above. Moreover, it appears desirable to reduce the amount of photosensitiser associated with the components of the reticulo-endothelial system, such as liver and spleen, since the apparently prolonged persistence of the C11Pc in such organs could induce some long-term toxicity effects. Therefore, while the use of gold nanoparticles for the conjugation and intravenous injection of C11Pc induces a greater selectivity of tumour targeting and a more efficient tumour response to PDT, the possibility to further optimise the efficacy of the phototherapeutic process by using suitably masked nanoparticles will be specifically investigated.

5. Conclusions

The pharmacokinetic data reveal that by conjugating the C11Pc photosensitiser to gold nanoparticles an enhanced accumulation within a sub-cutaneously implanted amelanotic melanoma is achieved as compared with the free phthalocyanine. Photodynamic therapy studies confirm that tumour growth is optimally slowed following light treatment 3 h post-i.v. injection. Electron microscopy studies have confirmed that the mechanism of photodamage of the tumour is via destruction of the vasculature. The data show that the use of nanoparticles for the delivery of hydrophobic photosensitisers, such as phthalocyanines, significantly enhances the PDT efficacy, even though suitable approaches should be devised in order to limit the persistence of the nanoparticle-associated photosensitiser in important organs such as liver and spleen.

Conflict of interest statement

None declared.

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REFERENCES

1. Dougherty TJ, Gomer CJ, Henderson BW, et al. Photodynamic therapy. *J Natl Cancer Inst* 1998;**90**:889–905.
2. Triesscheijn M, Baas P, Schellensa JHM, Stewarta FA. Photodynamic therapy in oncology. *The Oncologist* 2000;**11**:1034–44.
3. Juzeniene A, Peng Q, Moan J. Milestones in the development of photodynamic therapy and fluorescence diagnosis. *Photochem Photobiol Sci* 2007;**6**:1234–45.
4. O'Connor AE, Gallagher WM, Byrne AT. Porphyrin and Nonporphyrin photosensitisers in oncology: preclinical and clinical advances in photodynamic therapy. *Photochem Photobiol* 2009;**85**:1053–74.
5. Boyle RW, Dolphin D. Structure and biodistribution relationships of photodynamic sensitizers. *Photochem Photobiol* 1996;**64**:469–85.
6. Cinteza LO, Ohulchanskyy TJ, Sahoo Y, et al. Diacyllipid micelle-based nanocarrier for magnetically guided delivery of drugs in photodynamic therapy. *Mol Pharm* 2006;**3**:415–23.
7. Roy I, Ohulchanskyy TJ, Pudavar HE, et al. Ceramic-based nanoparticles entrapping water-insoluble photosensitising anticancer drugs: novel drug-carrier system for photodynamic therapy. *J Am Chem Soc* 2003;**125**:7860–5.
8. Chatterjee DK, Fong LS, Zhang Y. Nanoparticles in photodynamic therapy: an emerging paradigm. *Adv Drug Deliv Rev* 2008;**60**:1627–37.
9. Bechet D, Couleaud P, Frochot C, Viriot ML, Barberi-Heyob M. Nanoparticles for photodynamic therapy agent delivery. *Trends Biotechnol* 2008;**26**:612–21.
10. Moreno MJ, Monson E, Reddy RG, et al. Production of singlet oxygen by $Ru(dpp(SO_3)_2)_3$ incorporated in polyacrylamide PEBBLES. *Sens Actuators B-Chem* 2003;**90**:82–9.
11. Wang SZ, Gao RM, Zhou FM, Selke M. Nanomaterials and singlet oxygen photosensitisers: potential applications in photodynamic therapy. *J Mater Chem* 2004;**14**:487–93.
12. Gao D, Agayan RR, Xu H, Philbert MA, Kopelman R. Nanoparticles for two-photon photodynamic therapy in living cells. *Nano Lett* 2006;**6**:2383–6.
13. Moghimi SM, Hunter AC, Murray JC. Nanomedicine: current status and future prospects. *FASEB J* 2005;**19**:311–30.
14. Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. *Adv Drug Deliv Rev* 2004;**56**:1649–59.
15. Konan YN, Berton M, Gurny R, Allemann E. Enhanced photodynamic activity of meso-tetra(4-hydroxyphenyl)porphyrin by incorporation into sub-200 nm nanoparticles. *Eur J Pharm Sci* 2003;**18**:241–9.
16. McCarthy JR, Perez JM, Bruckner C, Weissleder R. Polymeric nanoparticle preparation that eradicates tumors. *Nano Lett* 2005;**5**:2552–6.
17. Gomes AJ, Lunardi LO, Marchetti JM, Lunardi CN, Tedesco AC. Photobiological and ultrastructural studies of nanoparticles of poly(lactic-co-glycolic acid)-containing bacteriochlorophyll-a as a photosensitizer useful for PDT treatment. *Drug Deliv* 2005;**12**:159–64.
18. Konan-Kouakou YN, Boch R, Gurny R, Allemann E. In vitro and in vivo activities of verteporfin-loaded nanoparticles. *J Control Release* 2005;**103**:83–91.
19. Tang W, Xu H, Kopelman R, Philbert MA. Photodynamic characterization and in vitro application of methylene blue-containing nanoparticle platforms. *Photochem Photobiol* 2005;**81**:242–9.
20. Zeisser-Labouebe M, Lange N, Gurny R, Delie F. Hypericin-loaded nanoparticles for the photodynamic treatment of ovarian cancer. *Int J Pharm* 2006;**326**:174–81.
21. Ricci Jr E, Marchetti JM. Preparation, characterization, photocytotoxicity assay of PLGA nanoparticles containing zinc(II) phthalocyanine for photodynamic therapy use. *J Microencapsul* 2006;**23**:523–38.
22. Ricci Jr E, Marchetti JM. Zinc(II) phthalocyanine loaded PLGA nanoparticles for photodynamic therapy use. *Int J Pharm* 2006;**310**:187–95.
23. Allemann E, Brasseur N, Benrezzak O, et al. PEG-coated poly(lactic acid) nanoparticles for the delivery of hexadecafluoro-zinc-phthalocyanine to EMT-6 mouse mammary-tumors. *J Pharm Pharmacol* 1995;**47**:382–7.
24. Yan F, Kopelman R. The embedding of meta-tetra(hydroxyphenyl)-chlorin into silica nanoparticle platforms for photodynamic therapy and their singlet oxygen production and pH-dependent optical properties. *Photochem Photobiol* 2003;**78**:587–91.
25. Davydenko MO, Radchenko EO, Yaschchuk VM, et al. Sensibilization of fullerene C-60 immobilized at silica nanoparticles for cancer photodynamic therapy. *J Mol Liq* 2006;**127**:145–7.
26. Mazzaglia A, Angelini N, Darcy R, et al. Novel heterotopic colloids of anionic porphyrins entangled in cationic amphiphilic cyclodextrins: spectroscopy investigation and intracellular delivery. *Chem Eur J* 2003;**9**:5762–9.
27. Sortino S, Mazzaglia A, Monsù Scolaro L, et al. Nanoparticles of cationic amphiphilic cyclodextrins entangling anionic porphyrins as a "carrier-sensitizer" system in photodynamic cancer therapy. *Biomaterials* 2006;**27**:4256–65.
28. Hone DC, Walker PI, Evans-Gowing R, et al. Generation of cytotoxic singlet oxygen via phthalocyanine–stabilized gold nanoparticles: a potential delivery vehicle for photodynamic therapy. *Langmuir* 2002;**18**:2985–7.

29. Wieder ME, Hone DC, Cook MJ, Handsley MM, Gavrilovic J, Russell DA. Intracellular photodynamic therapy with photosensitizer-nanoparticle conjugates: cancer therapy using a 'Trojan horse'. *Photochem Photobiol Sci* 2006;**5**:727–34.
30. Cheng Y, Samia AC, Meyers JD, et al. Highly efficient drug delivery with gold nanoparticle vectors for in vivo photodynamic therapy of cancer. *J Am Chem Soc* 2008;**130**:10643–7.
31. Reddy GR, Bhojani MS, McConville P, et al. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res* 2006;**12**:6677–86.
32. Sun Y, Chen ZL, Yang XX, et al. Magnetic chitosan nanoparticles as a drug delivery system for targeting photodynamic therapy. *Nanotechnology* 2009;**20**:AR 135102.
33. Hu ZY, Pan YF, Wang JW, et al. Meso-tetra (carboxyphenyl) porphyrin (TCPP) nanoparticles were internalized by SW480 cells by a clathrin-mediated endocytosis pathway to induce high photocytotoxicity. *Biomed Pharmacother* 2009;**63**:155–64.
34. Nishiyama N, Nakagishi Y, Morimoto Y, et al. Enhanced photodynamic cancer treatment by supramolecular nanocarriers charged with dendrimer phthalocyanine. *J Control Release* 2009;**133**:245–51.
35. Zhang M, Murakami T, Ajima K, et al. Fabrication of ZnPc/protein nanohorns for double photodynamic and hyperthermic cancer phototherapy. *Proc Natl Acad Sci* 2008;**105**:14773–8.
36. Taub AF. Photodynamic therapy in dermatology. In: Hamblin MR, Mroz P, editors. *Advances in photodynamic therapy: basic, translational and clinical*. Boston: Artech House; 2008. p. 419–42.
37. Soncin M, Busetti A, Reddi E, et al. Pharmacokinetic and phototherapeutic properties of axially substituted Si(IV)-tetradibenzobarreleno-octabutoxyphthalocyanines. *J Photochem Photobiol B: Biol* 1997;**40**:163–7.
38. Fabris C, Vicente MGH, Hao E, et al. Tumour-localizing and -photosensitising properties of meso-tetra(4-nido-carboranylphenyl)porphyrin. *J Photochem Photobiol B: Biol* 2007;**89**:131–6.
39. Reddi E, Zhou C, Biolo R, Menegaldo E, Jori G. Liposome or LDL-administered Zn(II)-phthalocyanine as a photodynamic agent for tumours. I. Pharmacokinetic properties and phototherapeutic efficiency. *Br J Cancer* 1990;**61**:407–11.
40. Ometto C, Fabris C, Milanesi C, Jori G, Cook MJ, Russell DA. Tumour-localising and -photosensitising properties of a novel zinc(II) octadecylphthalocyanine. *Br J Cancer* 1996;**74**:1891–9.
41. Fabris C, Ometto C, Milanesi C, et al. Tumour-localizing and tumour-photosensitizing properties of zinc(II)-octapentylphthalocyanine. *J Photochem Photobiol B: Biol* 1997;**39**:279–84.
42. Jori G. Photodynamic therapy: basic and preclinical aspects. In: Horspool W, Lenci F, editors. *Organic photochemistry and photobiology*. Boca Raton, FL: CRC Press; 2004. p. 146-1–146-10.
43. Jori G. Tumour photosensitisers: approaches to enhance the selectivity and efficiency of photodynamic therapy. *J Photochem Photobiol B: Biol* 1996;**36**:87–93.
44. Friedberg JS, Mick R, Stevenson J. A phase I study of Foscan-mediated photodynamic therapy in patients with mesothelioma. *Ann Thorac Surg* 2003;**75**:952–9.
45. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy. 2. Cellular signalling, cell metabolism and modes of cell death. *Photodiag Photodyn Ther* 2005;**2**:1–23.
46. Duncan R, Sat YN. Tumour targeting by enhanced permeability and retention effect. *Ann Oncol* 1998;**9**:39–52.
47. Paciotti GF, Kingston DGI, Tamarkin L. Colloidal gold nanoparticles: a novel nanoparticle platform for developing multifunctional tumour-targeted drug delivery vectors. *Drug Deliv Res* 2006;**67**:47–53.
48. Sharman WM, van Lier JE, Allen CM. Targeted photodynamic therapy via receptor mediated delivery systems. *Adv Drug Deliv Rev* 2004;**56**:53–76.
49. Nimjee SM, Rusconi CP, Sullenger BA. Aptamers: an emerging class of therapeutics. *Ann Rev Med* 2005;**72**:250–6.
50. Hou LC, Veeravagu A, Hsu AR. Recurrent gliobastoma multiforme: a review of natural history and management options. *Neurosurg Focus* 2006;**20**:5–11.
51. Carstensen H, Muller RH, Muller PW. Particle-size, surface hydrophobicity and interaction with serum of fat emulsions and model drug carriers as parameters related to RES uptake. *Clin Nutr* 1992;**11**:289–97.
52. Gbadamosi JK, Hunter AC, Moghimi SM. Pegylation of microspheres generates a heterogeneous population of particles with different surface characteristics and biological performance. *FEBS Lett* 2002;**532**:338–44.