Tumour-localizing and tumour-photosensitizing properties of zinc(II)-octapentyl-phthalocyanine

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Abstract

Zn(II)-octapentyl-phthalocyanine (ZnOPPc), incorporated into a Cremophor oil emulsion, was found to be a highly selective tumour-targeting agent (50-fold larger concentration in the tumour than in the peritumoral tissue) when injected at a dose of 1.46 μmol (kg body weight) $^{-1}$ in Balb/c mice bearing an intramuscularly transplanted MS-2 fibrosarcoma. The pharmacokinetic properties of this phthalocyanine were closely similar to those found for the analogous octadecyl derivative, whereas the unsubstituted Zn(II)-phthalocyanine showed a lower efficiency and selectivity of tumour targeting than the octaalkyl-substituted phthalocyanines. Irradiation of the ZnOPPc-loaded tumour with 620–700 nm light 24 h after injection caused a significant delay of tumour growth with a gradual shrinkage of the neoplastic mass; the damage involved important contributions from both random necrosis and apoptosis of malignant cells. © 1997 Elsevier Science S.A.

1. Introduction

Recently [1], we have described the pharmacokinetic and phototherapeutic properties of a newly synthesized phthalocyanine, namely Zn(II)-octadecyl-phthalocyanine (Zn-ODPc). In comparison with most phthalocyanines, including the parent compound Zn-phthalocyanine (ZnPc), ZnODPc exhibits an unusually high selectivity and efficiency of tumour targeting, an essentially complete absence of skin photosensitizing activity at about 12 h after administration and a shrinkage in tumour volume after PDT treatment without any detectable formation of tissue necrosis. Electron microscopy analyses of ZnODPc-photosensitized tumour specimens show an ultrastructural pattern, suggesting a frequent occurrence of cell apoptosis in addition to random cell death. We hypothesized that these peculiar features may be related to the large amount of ZnODPc found to be associated with serum low-density lipoproteins (LDLs), since these lipoproteins express an efficient interaction with several types of malignant cell through receptor-mediated endocytosis [2,3].

On the basis of such potentially promising observations, it is of interest to assess which structural properties determine the in vivo behaviour of ZnODPc. Therefore we have
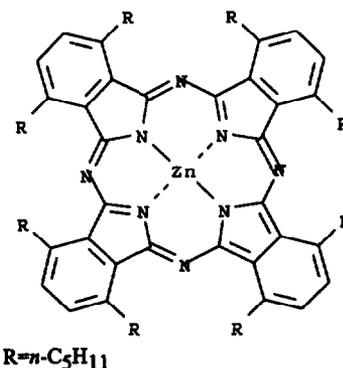

R=n-C₅H₁₁

Fig. 1. Chemical structure of ZnOPPc.

extended our previous investigations to a structural analogue of ZnODPc, namely Zn(II)-octapentyl-phthalocyanine (ZnOPPc, Fig. 1), where the alkyl side-chains have an intermediate length and bulkiness between ZnODPc and ZnPc.

2. Experimental section

2.1. Chemicals

The synthesis of zinc(II)-1,4,8,11,15,18,22,25-octakis-pentylphthalocyanine (ZnOPPc) has been described else-

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where [4]. This phthalocyanine is characterized by an absorption maximum located at 700 nm in tetrahydrofuran with a molar absorption coefficient (ϵ) of 2.06×10^5 dm^3 mol^{-1} cm^{-1}. ZnOPPc is hydrophobic; hence it was incorporated into an aqueous emulsion of Cremophor-EL for administration in vivo and its concentration was estimated by diluting a determined aliquot of the phthalocyanine-containing emulsion into a known excess of tetrahydrofuran and reading the absorbance at 700 nm.

Sodium dodecylsulphate (SDS) and tetrahydrofuran were purchased from Merck and were used as received. All other solvents and chemicals were commercial products of at least analytical grade.

2.2. Animals and tumour

Female Balb/c mice (20–22 g body weight), bearing an MS-2 fibrosarcoma intramuscularly transplanted into the right hind leg, were used throughout this investigation. The mice were purchased from Charles River (Como, Italy) and housed in standard cages with free access to tap water and normal dietary food. The mice were treated according to the guidelines established by the Italian Committee for Humane Care of Experimental Animals.

Tumour implantation was performed by injection of a sterile aqueous suspension (0.2 ml) of 10^6 cells of MS-2 fibrosarcoma, and no spontaneous regression or remission of the tumour was detected.

2.3. Pharmacokinetic studies

On the seventh day after transplantation, when the tumour diameter was 0.6–0.8 cm, the mice were injected with 1.46 μmol of Cremophor-incorporated ZnOPPc per kilogram body weight into the tail vein. At pre-determined times after administration, groups of three mice were sacrificed by prolonged exposure to ether vapour. The tumour and selected non-cancerous (normal) tissues were rapidly excised, washed with phosphate-buffered saline and assayed for the phthalocyanine content by spectrofluorometric analysis, after chemical extraction according to the procedure described previously [1]. At the same time, blood samples (approximately 1.0 ml per mouse) were taken intracardially from the sacrificed animals, centrifuged at 3000 rev min^{-1} for 15 min in order to remove the blood cells and the serum thus collected was pooled and tenfold diluted with 2% aqueous SDS and tetrahydrofuran. Both the serum and tissue extracts were assayed for the ZnOPPc content by reading the 633 nm excited phthalocyanine fluorescence emission in the 650–830 nm spectral interval; the corrected fluorescence maximum of ZnOPPc in cyclohexane was found to be located at 720 nm using 4-methylamino-4'-nitrostilbene in o-dichlorobenzene as a standard. The fluorescence intensity was converted into the ZnOPPc concentration by interpolation with a calibration plot.

In a different set of experiments, healthy mice were injected with 0.37, 0.73 and 1.46 μmol of ZnOPPc incorporated into a Cremophor-EL emulsion. At 24 h after intravenous injection, the mice were sacrificed and 2 ml of pooled serum was subjected to discontinuous density gradient ultracentrifugation in order to isolate the following serum protein fractions: very low-density lipoprotein (VLDL), LDL, high-density lipoprotein (HDL) and heavy proteins ($d > 1.2$ g/ml, mainly globulins and albumin). We have previously shown [5] that the distribution of phthalocyanine photosensitizers between mouse serum proteins is not affected by the presence of intramuscular MS-2 fibrosarcoma.

After ultracentrifugation, 0.4 ml of the individual serum protein fractions was added to 0.4 ml of 4% aqueous SDS and diluted with 1.5 ml of tetrahydrofuran. The amount of ZnOPPc associated with the different fractions was determined by spectrofluorometric analysis.

2.4. Phototherapeutic studies

Irradiation of the tumour-bearing mice was performed 24 h after administration of 1.46 μmol kg^{-1} of ZnOPPc using 620–700 nm light isolated from the emission of a quartz/halogen lamp (Teclas, Lugano, Switzerland) by optical filtering. The lamp was operated at 230 mW cm^{-2} for a total delivered light dose of 400 J cm^{-2}. The irradiation procedure has been described elsewhere [5].

The tumour response was analysed by visual observation, by measurement of the tumour volume at daily intervals after the end of PDT and by electron microscopy studies on tumour specimens taken at pre-determined post-irradiation times (at least three mice per time were examined). The preparation of the samples and the ultrastructural determinations were performed as described previously [5]. The volume of the tumour was calculated using the formula $V = 2/3\pi(a/2 \times b/2 \times c)$, where a, b and c represent the three mutually perpendicular directions measured with a calliper; this assumes a hemiellipsoidal structure for the volume. The data were compared with the tumour volume found for control mice that had been transplanted simultaneously with the photo-treated mice but had not been injected with the photosensitizer and exposed to light. In parallel experiments, healthy Balb/c mice receiving 1.46 μmol kg^{-1} of ZnOPPc were irradiated on the right hind leg at 3 h and 48 h after intravenous injection of the phthalocyanine in order to estimate the skin photosensitivity. The light source was operated at a fluence rate of 230 mW cm^{-2} and the total light dose was 300 or 400 J cm^{-2}. The skin response to the phototreatment was estimated by visual observation at daily intervals for about 1 week after irradiation.

3. Results

3.1. Pharmacokinetic studies

The distribution of ZnOPPc between the mouse plasma proteins is shown in Table 1 for three different injected doses.

Table 1

Distribution of ZnOPPc between mouse plasma proteins 24 h after intravenous administration of the phthalocyanine in a Cremophor-EL emulsion. Protein fractions were separated by density gradient ultracentrifugation and the amount of associated phthalocyanine was measured by spectrofluorometric analysis

Injected dose (μmol kg^{-1})	Percentage recovery				
	VLDL	LDL	HDL	H.P.	Cremophor
1.46	n.d.	61.7	20.3	2.2	15.8
0.73	n.d.	56.4	33.7	3.7	6.2
0.37	n.d.	23.1	62.4	5.1	9.4

H.P., heavy proteins; n.d., not detected.

The analysis was performed at 24 h after intravenous administration of the phthalocyanine owing to the well-known low rate at which Cremophor-incorporated photosensitizers are released to serum proteins [6]; this was further confirmed by the observation that, even after 24 h, approximately 10% of the total ZnOPPc in the serum was still associated with the oil emulsion. However, it is apparent that at least 85% of the recovered ZnOPPc is bound by the lipoprotein fraction, although the partitioning of ZnOPPc between HDL and LDL depends on the phthalocyanine dose. It is important to underline that, at this time, only about 10% of the ZnOPPc recovered in the serum at 1 h post-injection is still present in the mouse serum (see Table 2).

Table 2 also shows the time dependence of ZnOPPc distribution in the tumour, muscle (which represents the peritumoral tissue in our animal model) and selected healthy tissues. The largest phthalocyanine concentrations were found in the components of the reticuloendothelial system, typical of hydrophobic porphyrinoids which are administered in association with lipid vehicles [7] and are cleared from the organism via the bile-gut pathway [8]. On the other hand, relatively small amounts of ZnOPPc are accumulated and rapidly cleared from other normal tissues, such as kidneys and lung. No detectable amounts of phthalocyanine were recovered from brain tissue at all the post-injection times examined (data not shown); this finding suggests that Zn-

OPPc is unable to cross the blood–brain barrier, similar to the observations previously made for unsubstituted ZnPc [8].

3.2. Phototherapeutic studies

Irradiation of the fibrosarcoma was performed 24 h after ZnOPPc injection, since maximal phthalocyanine concentrations in the tumour were reached at this time. The phototreated area became extensively oedematous within 24 h after the end of PDT, and the tumour growth rate was significantly lower than that typical of unirradiated control mice: the growth time (i.e. the time interval for the tumour to grow to a volume of 0.3 cm^3 from the volume of 0.04 cm^3 measured immediately before irradiation) was 14.8 ± 2.3 days for the phototreated tumour compared with 8.2 ± 1.9 days for the controls (average of five animals). However, no formation of eschar or other necrotic features was observed, contrary to the observations made when the same tumour model was photosensitized with other lipophilic or hydrophilic dyes [9].

The nature of the photoinduced tissue damage was further analysed by electron microscopy. At 1 h after PDT, neoplastic cells show swollen or optically empty mitochondria with an average increase in the intracellular spaces (Fig. 2). The direct damage of neoplastic cells becomes more pronounced after 3 h (Fig. 3) with a dilation of the cisternae of the endoplasmic reticulum, the appearance of extensive vacuolization in the cytoplasm and the swelling or detachment of the perinuclear membrane. On the other hand, endothelial cells are still rather well preserved at this post-irradiation time, except for the swelling of some mitochondria which display a partially altered matrix (Fig. 4). The necrosis of the tumour tissue becomes quite extensive 24 h after irradiation; as shown in Fig. 5, the cell borders can be barely detected and the overall architecture of the tissue is highly disorganized; we can observe some finely dispersed granular material, and the cytoplasm has been converted to large groups of inhomogeneous vesicles.

The pattern described above is also associated with less frequent features, such as the presence of several granulocytes in the interstitial areas of the tumour tissue at short post-

Table 2

Recovery (nanomoles of dye per gram of tissue) of ZnOPPc from serum and selected tissues of Balb/c mice bearing an intramuscularly transplanted MS-2 fibrosarcoma. The mice were injected intravenously with 1.46 μmol kg^{-1} of ZnOPPc solubilized in a Cremophor-EL emulsion

| Tissue	Recovery (nmol g^{-1})						
	1 h	3 h	6 h	15 h	24 h	48 h	1 week
Tumour	1.81 ± 0.39	1.83 ± 0.13	2.05 ± 0.31	2.10 ± 0.34	2.42 ± 0.32	1.86 ± 0.06	0.63 ± 0.01
Muscle	0.10 ± 0.02	0.06 ± 0.00	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.03 ± 0.01	n.d.
Skin	0.29 ± 0.06	0.22 ± 0.07	0.17 ± 0.08	0.15 ± 0.03	0.15 ± 0.02	0.22 ± 0.01	0.13 ± 0.04
Liver	2.28 ± 0.19	3.30 ± 0.15	4.41 ± 0.25	6.50 ± 0.37	6.55 ± 0.80	7.95 ± 1.19	6.07 ± 0.65
Spleen	0.93 ± 0.05	1.09 ± 0.11	2.24 ± 0.38	3.24 ± 0.12	3.30 ± 0.12	2.77 ± 0.27	1.69 ± 0.33
Kidney	1.69 ± 0.31	1.49 ± 0.16	1.09 ± 0.14	0.56 ± 0.02	0.40 ± 0.01	0.11 ± 0.02	0.06 ± 0.00
Lung	2.50 ± 0.03	1.60 ± 0.17	1.45 ± 0.19	0.35 ± 0.12	0.25 ± 0.07	0.05 ± 0.02	n.d.
Serum [a]	18.11 ± 0.62	11.28 ± 1.23	9.10 ± 1.04	4.79 ± 0.33	2.57 ± 0.37	0.22 ± 0.03	n.d.

[a] Unit: nanomoles per millilitre; n.d., not detected.
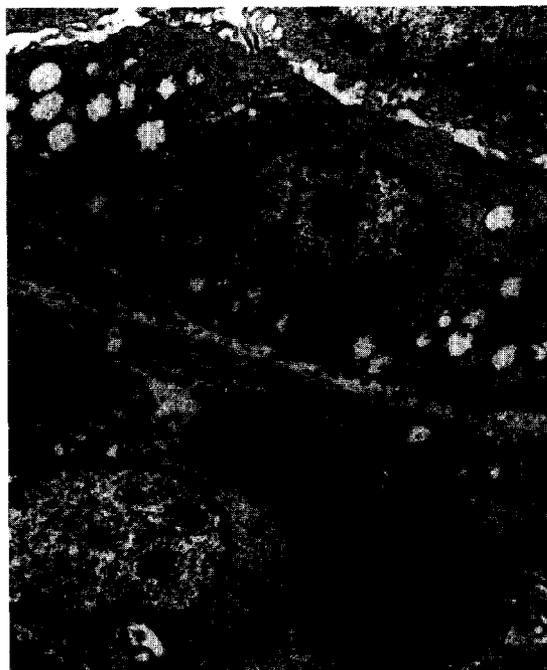
Fig. 2. Tumour specimen obtained 1 h after PDT (× 6500). The ultrastructural features differ from those typical of control tumours with an average increase in the intracellular spaces; the mitochondria (m) of the neoplastic cells are significantly swollen and optically empty, but the endothelial cells show a generally well-preserved ultrastructure.
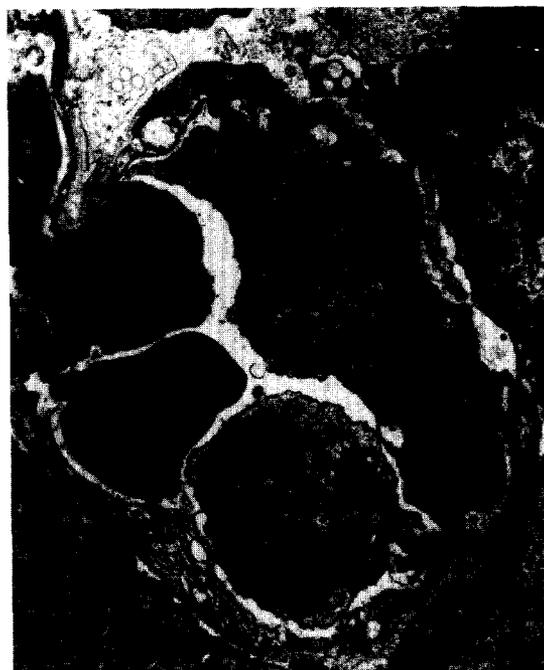

Fig. 4. Tumour specimen obtained 3 h after PDT (× 8500). The endothelial cells are still rather well preserved, except for the swelling of some mitochondria (m) and a partial alteration of their matrix.
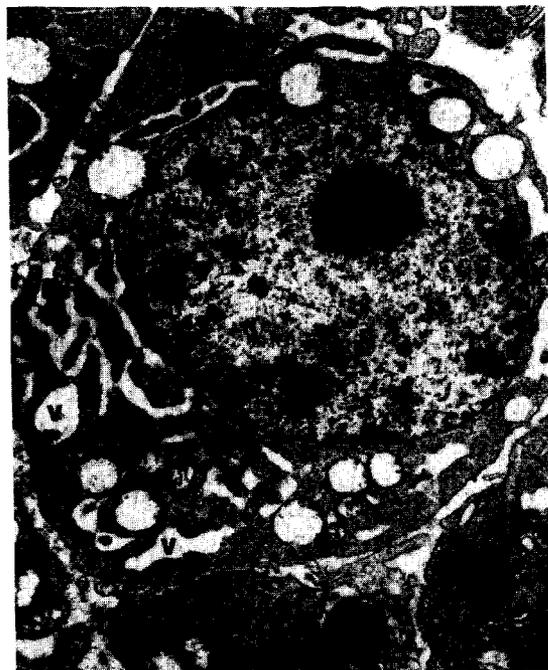

Fig. 3. Tumour specimen obtained 3 h after PDT (× 9000). The damage of neoplastic cells is more pronounced with dilation of the cisternae of the endoplasmic reticulum (ER) and the formation of numerous cytoplasmic vacuoles (v). The nucleus shows a swelling or detachment of the perinuclear membrane (arrows).

Fig. 5. Tumour specimen obtained 24 h after PDT (× 6000). The organization of the tumour tissue is almost completely lost and the damage involves all the subcellular structures with disruption of the plasma membrane and evidence of karyorrhexis.

irradiation times (Fig. 6); this event represents a typical consequence of irritative stimuli.

Lastly, several micrographs display ultrastructural features which indicate the occurrence of PDT-induced apoptosis. As typically shown in Fig. 7, some neoplastic cells are characterized by condensation of chromatin at the nuclear periphery,
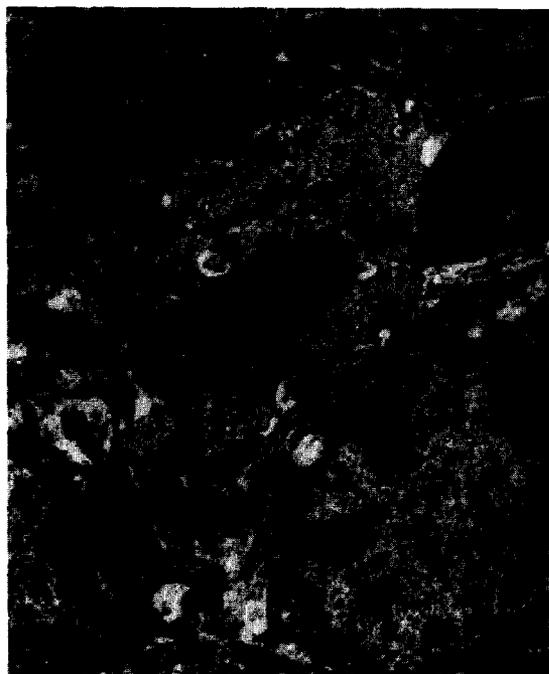
Fig. 6. Tumour specimen obtained 3 h after PDT (× 7000). The micrograph shows the presence of several granulocytes (G) in the interstitial areas of the tumour tissue.
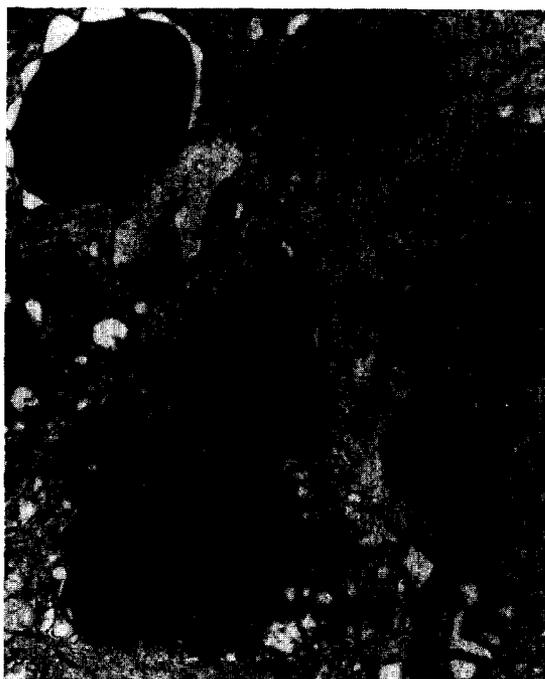

Fig. 7. Tumour specimen obtained 3 h after PDT (× 12 000). The micrograph shows ultrastructural features typical of apoptosis: heavily condensed chromatin at the nuclear periphery and marked swelling of the perinuclear membrane with the formation of blebs (arrows).

swelling of the perinuclear membrane and the formation of blebs.

In a different set of experiments, the possible generation of skin photosensitivity by ZnOPPc was tested 3 h and 48 h

after injection of 1.46 μmol kg^{-1}. The irradiation protocols are described in Section 2. In all three mice irradiated at 3 h, we observe the formation of oedema within about 8 h after the end of the phototreatment; this effect is more pronounced for irradiation with 400 J cm^{-2}. However, no detectable skin photodamage is induced by irradiation at 48 h. This observation confirms our previous findings with ZnODPc [1] which indicate that cutaneous photosensitivity is associated with the serum concentration of the photosensitizer rather than with its concentration in the skin.

4. Discussion

The pharmacokinetic properties of Cremophor-delivered ZnOPPc, after systemic injection to fibrosarcoma-bearing mice at a dose of 1.46 μmol kg^{-1}, closely resemble those previously observed for ZnODPc in the same experimental model [1]. Thus the maximal amount of photosensitizer accumulation in the tumour is essentially identical for the two phthalocyanines, and the ratio of ZnOPPc or ZnODPc concentration in the tumour to the peritumoral tissue is about 50 or larger 24–48 h after administration; this indicates an excellent selectivity of tumour targeting (see Table 2). These parameters are clearly more favourable than those typical of unsubstituted ZnPc which, under similar experimental conditions, yields a lower efficiency of tumour targeting (about 30% smaller) and a tumour to muscle ratio of photosensitizer concentration of about seven [5]. Although a detailed explanation of the differences in the pharmacokinetic properties between the unsubstituted and polyalkylated phthalocyanines cannot be given at this stage, we can possibly correlate the behaviour of ZnODPc and ZnOPPc with the unusually large amount of phthalocyanine which is bound to the LDL fraction of serum proteins (60%–70% vs. about 25% for ZnPc). Therefore the present findings lend further support to the importance of the LDL pathway for the transport of hydrophobic photosensitizing agents to a variety of solid tumours [3,10]. Interestingly, the serum distribution data reported in Table 1 appear to indicate that the association of ZnOPPc with LDL is favoured by the injection of relatively large phthalocyanine doses, whereas for lower ZnOPPc amounts preferential binding to HDL takes place. At the present stage of our investigations, we can only formulate some hypotheses to explain such observations. One interpretation could be based on the intrinsically higher affinity of ZnOPPc for HDL; interaction should occur with saturable binding sites, e.g. sites located in the apoprotein moiety [11], which is appreciably more important in HDL than in other lipoproteins [12], although the long alkyl chains protruding from the phthalocyanine macrocycle should favour the incorporation of the photosensitizer into the lipid core of HDL. Alternatively, the LDL could release the associated phthalocyanine more rapidly than HDL, e.g. by delivering it to the tumour cells; thus the decrease in the residual amount of LDL-bound phthalocyanine would appear to be more evident on administration

of smaller doses. Our serum distribution analyses were performed 24 h post-injection, when the phthalocyanine recovery from the fibrosarcoma reaches maximum values. Unfortunately, as mentioned in Section 3, it was not possible to assay the partitioning of ZnOPPc between serum proteins at shorter post-injection times owing to the slow release of phthalocyanines from Cremophor emulsions.

In any case, the tumour-localized ZnOPPc is clearly endowed with efficient phototherapeutic activity. The mechanism of photoinduced tumour damage is similar to that observed for ZnODPc, since there is no indication of massive haemorrhagic necrosis as found for ZnPc [5]. Rather, gradual shrinkage of the neoplastic mass takes place and appears to involve both random cell death and cell apoptosis to similar extents, as suggested by electron microscopy studies on irradiated tumour specimens. While the ability of several photodynamic sensitizers to induce the irreversible alteration of different cell organelles, especially membranous systems, has been assessed over a long time [13], the onset of apoptotic processes in malignant cells as a consequence of PDT treatment has been reported only recently [14–16]. The phenomenon does not seem to be of general occurrence [17]. In most cases, including tumour photosensitization by ZnPc, the contribution of apoptosis to the overall photodamage is of limited frequency and importance compared with random necrosis. Thus our findings indicate that the introduction of alkyl substituents at the periphery of the tetraazaisoindole macrocycle of phthalocyanines enhances the probability of photoinduced cell apoptosis. We are presently investigating whether this reflects the localization of the polyalkylated phthalocyanine in specific sites of the cell membrane, whose photosensitized modification can promote the cascade of events (e.g. phospholipase activation) which eventually leads to programmed cell death [18].

Acknowledgements

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