Liposome- or LDL-administered Zn (II)-phthalocyanine as a photodynamic agent for tumours. I. Pharmacokinetic properties and phototherapeutic efficiency

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Summary The pharmacokinetics of Zn-phthalocyanine (Zn-Pc) in mice bearing a transplanted MS-2 fibrosarcoma has been studied using dipalmitoyl-phosphatidylcholine (DPPC) liposomes and low density lipoproteins (LDL) as drug delivery systems. LDL induce a higher Zn-Pc uptake by the tumour and improve the selectivity of tumour targeting as compared to DPPC liposomes. Experimental photodynamic therapy (PDT) of the MS-2 fibrosarcoma has been performed using liposome-delivered Zn-Pc and the efficiency of tumour necrosis has been measured following four different irradiation protocols. We found that Zn-Pc doses as low as 0.07–0.35 mg kg\(^{-1}\) are sufficient for inducing an efficient tumour response that is linearly dependent on the injected dose. The amount of Zn-Pc in the tumour decreases very slowly as a function of time, hence PDT gives satisfactory results even if performed at relatively long time intervals after administration.

In this paper we report a comparative pharmacokinetic study of Zn-Pc injected into tumour-bearing mice after incorporation into DPPC liposomes (Zn-Pc-DPPC) or in vitro complexation to human LDL (Zn-Pc-LDL). PDT has been performed after the administration of Zn-Pc-DPPC and the extent of the photoinduced tumour necrosis has been measured as a function of different experimental parameters.

Materials and methods

Chemicals
Zn-Pc was a gift from Ciba-Geigy (Basel, Switzerland) and has been used as received. Previous analysis of the compound showed a degree of purity of 97% (Valduga et al., 1987). D,L-α-DPPC was a product of Sigma Chemical Co. All other chemicals and solvents were analytical grade reagents.

Animals and tumour
Female Balb/c mice (18–20 g body weight) obtained from Charles River (Como, Italy) have been used as experimental model. The mice were grown in cages with free access to standard dietary chow and tap water. Animal care was made according to the guidelines established by the Italian committee for experiments on animals. The MS-2 fibrosarcoma has been supplied by Istituto Nazionale dei Tumori, Milan. The tumour was implanted in the right hind leg of the mice by injection of 0.25 ml of a cell suspension containing at least 10\(^6\) cells ml\(^{-1}\). All the experiments were started at 8 days after tumour implantation, when its diameter was 0.7–0.8 cm. When necessary, the mice were anaesthetised by i.p. injection of ketalar (150 mg kg\(^{-1}\)).

Drug preparation
Zn-Pc was incorporated into small unilamellar vesicles of DPPC following the procedure described by Valduga et al. (1987). For these experiments liposomes were prepared in 0.9% aqueous NaCl and before injection they were dialysed for 3 h against 250 ml of NaCl with a change of the NaCl solution after the first hour.

The Zn-Pc was complexed in vitro with human LDL after isolation of this lipoprotein fraction (1.006 < \(d\) < 1.063) by density gradient ultracentrifugation (Havel et al., 1955) of blood samples obtained from normal lipidaemic volunteers. The isolated LDLs were dialysed overnight at 4°C, against 0.01 M phosphate buffer, pH 7.4, with a change of the buffer...
after the first hour. The Zn-Pc was incorporated into LDL by the slow solution of the drug in pyridine, which had been 5-fold diluted with absolute ethanol, to an aqueous solution, pH 7.4, of LDL (7 mg ml⁻¹). The added volume of the Zn-Pc solution was one-tenth the volume of the lipoprotein solution. During the addition, the lipidoprotein solution was kept at 37°C. In order to remove the organic solvents, the Zn-Pc-LDL complex was dialysed at room temperature for 3 h against phosphate buffer at pH 7.4 with one change of the buffer after the first hour.

The Zn-Pc concentration in the liposomal vesicles or in the lipoproteins was measured by absorbance at 673 nm using ε = 2.41 x 10⁴ M⁻¹cm⁻¹ in pyridine (value determined in our laboratory).

Pharmacokinetic studies

The Zn-Pc-DPPC or the Zn-Pc-LDL were injected i.v. into tumour-bearing mice at a dose of 0.12 mg kg⁻¹. At different times after injection the animals were killed, serum samples as well as the tumour and several normal tissues were collected for the analysis of their Zn-Pc content. The tissues were washed with saline solution and frozen until the analysis was performed. The concentration of Zn-Pc in the serum and in the tissues was measured spectrophotofluorimetrically following the procedure previously described by Reddi et al. (1987). The assay method was essentially based on tissue homogenisation in 2% SDS and measurement of the fluorescence emission intensity of Zn-Pc in the supernatant which was obtained after centrifugation of the homogenate. This procedure allows an essentially quantitative extraction of the drug from tissues (Reddi et al., 1987).

Photodynamic therapy

The experimental PDT with Zn-Pc-DPPC was performed by irradiation of the tumour area with red light (590–750 nm) isolated from a 250 W halogen lamp equipped with a set of optical filters. The emitted light was focused into a bundle of optical fibres having a total diameter of 0.6 cm. The fibre tip was placed at 1 cm from the tumour surface during the phototreatments. Tumour irradiation was performed according to different protocols (Table I) in order to examine the effect of various experimental parameters on the extent of the photoinduced tumour necrosis.

The estimation of the extent of tumour necrosis was performed at 24 h after PDT. The procedure adopted involved the fixation of the tumour in 4% formalin, followed by sectioning of the tumour at 2 mm intervals. The width and depth of the necrotic area were measured for each tissue slice. The maximum values of width and depth were recorded for each tumour and their product was chosen for a quantitative evaluation of the tumour response. Each point, reported in the figures describing the PDT data, represents the mean (± s.d.) from at least three animals.

Results

Pharmacokinetic studies

Serum samples taken from mice at various times after i.v. injection of Zn-Pc were analysed by column chromatography as previously described (Reddi et al., 1987). Both DPPC- and LDL-administered Zn-Pc were found to be exclusively associated with the lipoprotein fraction, similar to what we have observed for i.p.-injected Zn-Pc-DPPC (Reddi et al., 1987). The elimination of Zn-Pc from the serum follows a biphasic kinetics. About 70% of the drug is eliminated in about 12 h while the remaining fraction is eliminated rather slowly: Zn-Pc levels as low as approx. 40 ng ml⁻¹ are present in the serum at 7 days after the i.v. injection. The kinetics of Zn-Pc clearance from the serum are independent of the modality used for administration, i.e. via liposomes or LDL.

In Figures 1 and 2 we show the time-dependence of Zn-Pc concentration in selected tissues after i.v. administration of Zn-Pc-DPPC and Zn-Pc-LDL, respectively. The recoveries were estimated for the tumour, the muscle, i.e. the tissue where the tumour is implanted, and the liver, i.e. the normal tissue which usually shows a large uptake of systemically injected photosensitisers (Dougherty, 1984). Each experimental point represents the average of at least three independent determinations performed on specimens obtained from different animals. In the case of Zn-Pc-DPPC the maximum concentration of drug in the tumour was reached by 3 h after administration (Figure 1), while for the Zn-Pc-LDL about a two-fold greater maximum accumulation is observed at about 24 h (Figure 2). On the other hand, rather similar recoveries of Zn-Pc from the liver and muscle are obtained upon injection of Zn-Pc-DPPC and Zn-Pc-LDL.

The degree of selectivity of drug localisation in the tumour is usually expressed by the ratio of its concentration in the tumour and selected normal tissues. In Table II we report the ratios tumour/liver and tumour/muscle for Zn-Pc at different times after injection.

![Figure 1](image1.png)

**Figure 1** Time dependence of Zn-Pc concentration in tumour (●), liver (○) and muscle (△) of mice injected with 0.12 mg kg⁻¹ Zn-Pc-DPPC.

![Figure 2](image2.png)

**Figure 2** Time dependence of Zn-Pc concentration in tumour (●), liver (○) and muscle (△) of mice injected with 0.12 mg kg⁻¹ Zn-Pc-LDL.

| Protocol | Injected Zn-Pc dose (mg kg⁻¹) | Irradiation dose rate (mW cm⁻²) | Total light dose (J cm⁻²) | Δt (h) |
|----------|-------------------------------|-------------------------------|--------------------------|--------|
| 1        | 0.14                          | 50–200                        | 300                      | 24     |
| 2        | 0.14                          | 180                           | 150–450                  | 24     |
| 3        | 0.14                          | 180                           | 300                      | 3–72   |
| 4        | 0.07–0.35                     | 180                           | 300                      | 24     |

Δt is the time interval between the Zn-Pc injection and PDT.
Experimental photodynamic therapy

Generally, the tumours irradiated with red light after the i.v. injection of Zn-Pc-DPPC showed the appearance of a dark spot within a few hours, which gradually evolved into eschar formation and loss of part of the tumour mass. In all the experiments reported here, the extent of tumour response, as a function of different parameters, has been evaluated at 24 h after irradiation, i.e. before the loss of any tumour mass.

In Figure 3 we show the extent of the tumour necrosis as a function of the irradiation dose rate (Table I, protocol 1). Clearly, the tumour necrosis increases upon increasing the dose rate. With our light source, tumour necrosis was observed also in mice not injected with Zn-Pc but only upon irradiation with dose rates higher than 230 mW cm\(^{-2}\), possibly owing to the onset of thermal damage. The extent of the necrotic area depends also on the total light dose as shown by the data obtained with mice irradiated following protocol 2 (Figure 4). It appears that, under our conditions, only light doses above 150 J cm\(^{-2}\) induce detectable tumour necrosis. For a fixed dose rate and total light dose (protocol 3), the tumour necrosis is dependent on the time interval between drug administration and PDT (Figure 5). Significant tumour necrosis is observed for irradiation performed up to 70 h after the Zn-Pc injection, which is in agreement with the slow clearance of Zn-Pc from the tumour demonstrated by our pharmacokinetic studies. PDT was not performed at time intervals longer than 70 h after Zn-Pc injection, because of the too large tumour dimensions and the appearance of spontaneous necrosis. It is also clear from Figure 6 that a linear relationship exists between the extent of the photoinduced necrotic area and the injected dose of photosensitiser. Such a behaviour is not surprising owing to the low Zn-Pc doses used in our experiments. The upper limit of the photosensitiser dose tested by us was 0.35 mg kg\(^{-1}\), since at this dose the photoinduced necrosis involved the whole tumour mass.

Discussion

Our data show that relatively large Zn-Pc concentrations can be complexed "in vitro" with isolated LDL; thus, this lipoprotein class can be used "in vivo" as a Zn-Pc delivery system. The occurrence of an active LDL-receptor pathway for the delivery of Zn-Pc to our tumour model is suggested by the about two-fold larger concentrations of phthalocyanine observed in the tumour after injection of the Zn-Pc-DPPC compared with Zn-Pc-DPPC. However, even with LDL as a carrier, the selectivity of tumour targeting by Zn-Pc is limited by the fact that LDL-bound Zn-Pc once injected undergoes a partial redistribution among other lipoprotein classes, mainly high density lipoproteins (data not shown). The actual mechanism of Zn-Pc delivery from LDL (and/or other carriers) to the tumour tissue is certainly complex and probably involves the time-dependent redistribution of the dye among different tissue compartments. A similar behaviour has been observed for sulphonated porphine derivatives and HpD components (Kessel et al., 1987; Kessel, 1986b). This might

Table II: Ratio between the concentration of Zn-Pc in the tumour and liver and in the tumour and muscle for Zn-Pc-DPPC and Zn-Pc-LDL.

| Time (h) | Tumour/liver | Tumour/muscle |
|---------|--------------|---------------|
|         | Zn-Pc-DPPC   | Zn-Pc-LDL     | Zn-Pc-DPPC | Zn-Pc-LDL |
| 3       | 0.45         | 0.47          | 2.82       | 2.89      |
| 12      | 0.70         | 1.25          | 3.30       | 4.37      |
| 24      | 0.94         | 1.85          | 3.71       | 5.71      |
| 48      | 1.01         | 1.32          | 2.80       | 4.95      |
| 168     | 1.04         | 2.27          | 3.84       | 4.20      |
explain the lower efficiency of PDT at 3 h as compared to 24 h after Zn-Pc injection (Figure 5) in spite of the almost identical overall concentration of the photosensitisers in the tumour (Figures 1-3).

In any case, it is likely that Zn-Pc, once released inside the tumour cell, becomes associated with apolar compartments owing to its hydrophobic character. Ultrastructural studies on mouse tumour tissues, which had been irradiated in the presence of Zn-Pc, showed that the cytoplasmic and mitochondrial membranes were heavily damaged (Milanese et al., 1987). This endocellular distribution of Zn-Pc can explain the slow release of this drug from the tumour. We have previously demonstrated that Zn-Pc is eliminated from the body by the bile–gut pathway (Reddi et al., 1987). This requires the removal of Zn-Pc from the tumour and its transport to the liver by serum proteins; thus the slow clearance of Zn-Pc may depend on the low accessibility of the drug binding sites to the protein carriers. The poor lymphatic drainage typical of tumour tissues has also been invoked to explain the slow release of photosensitising drugs (Bugelski et al., 1981). The high degree of selectivity of tumour targeting by our procedure for Zn-Pc administration and transport is underlined by the fact that at 24 h after injection similar or larger amounts of Zn-Pc are found in the tumour as compared to the liver (see Figures 1 and 2).

Moreover, the constantly low levels of Zn-Pc in the muscle should indicate that there is only a small passage to normal tissues adjacent to the tumour during PDT.

The slow clearance of Zn-Pc by the tumour also suggests the possibility of multiple phototreatments following a single Zn-Pc injection. Our PDT data show that irradiations performed at 70 h after Zn-Pc administration induce an efficient tumour necrosis and, on the basis of our pharmacokinetic data (Figure 1), it is likely that similar tumour response are obtained with Zn-Pc after it has been administered for 7 days after Zn-Pc injection. These considerations are reinforced by the finding (Figure 6) that Zn-Pc doses as low as 0.07 mg kg⁻¹ are sufficient for inducing an important tumour necrosis. The linearity of the plot of the tumour responses against Zn-Pc dose (Figure 6) is due to the low amounts of photosensitising agent used in our experiments. On the other hand, Tralau et al. (1987b) found that the extent of tumour response to PDT tends toward a plateau value upon injection of sulphonated Al-Pc doses above 1 mg kg⁻¹. It is likely that in the presence of large dye concentrations in the tumour the incident light is efficiently absorbed only by the superficial layers of the tissue, thus reducing the optical penetration depth.

The extent of the tumour necrosis for a given Zn-Pc dose depends on the total light dose (see Figure 4), as already observed by other authors for porphyrins (Fingar et al., 1987) and phthalocyanines (Tralau et al., 1987b) and expected on the basis of theoretical considerations (Doiron et al., 1984). The extent of tumour necrosis depends also on the irradiation dose rate (see Figure 3). In particular, we observed a 6-fold increase of the necrotic area from 50 to 100 mW cm⁻². For normal tissues no tumour necrosis could be detected up to 230 mW cm⁻². Moreover, upon irradiation of Zn-Pc-injected mice with dose rates around 180 mW cm⁻², the temperature increase of the tumour tissue, as measured with an infra red sensitive telecamera (Vietri et al., 1988), was below 3–4°C, which is lower than that usually considered to originate hyperthermal effects (Evensen & Moan, 1988). However, a synergism between this modest temperature increase and photodynamic effects cannot be ruled out. The occurrence of such a synergism might explain the upward deviation from linearity observed above about 130 mW cm⁻² in the plot describing the influence of dose rate on the extent of the necrotic area (Figure 3).

Our pharmacokinetic studies show that a fraction of the Zn-Pc is cleared from the serum at a slow rate; this circumstance may be of some risk of drug toxicity, as the presence of Zn-Pc may lead to damage to normal tissues adjacent to the tumour.

In conclusion Zn-Pc appears to be a very promising photodynamic agent for the therapy of tumour due to its selective localisation in and slow clearance from the tumour, and the efficient photosensitisation of tumour necrosis even upon injection at very low doses.

This work received financial support from Consiglio Nazionale delle Ricerche, Italy, under the special project ‘Oncologia’, contract no. 87.01322.44.

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