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The Effect of Different Liposomal Formulations on the Interaction of Zn(II)-Phthalocyanine with Isolated Low and High Density Lipoproteins

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Several phthalocyanines exhibit a high affinity for tumour tissues and upon red-light irradiation originate a photosensitized process leading to tumour necrosis. This study was designed to define the role of different liposomal formulations in modulating the affinity of Zn(II)-phthalocyanine (ZnPc) for isolated low- and high-density lipoproteins (LDL, HDL). This information is important as the uptake of hydrophobic photosensitizers by tumour tissues can be enhanced by their association with LDL. The kinetics and efficiency of ZnPc association with LDL and HDL as well as the redistribution of ZnPc between the lipoproteins were studied by salt gradient ultracentrifugation and spectrophotometric analyses of the isolated lipoproteins. The formation of the photosensitizer–lipoprotein complexes in the plasma is affected by the vehicle utilized for the drug delivery. ZnPc in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-L-serine and 1,2-dimyristoyl-sn-glycero-3-phosphocholine liposomes is transferred to lipoproteins within a few minutes, while the transfer of ZnPc from 1,2-dipalmitoyl-sn-glycero-3-phosphocholine liposomes reaches a steady state value in a time scale of 5–6 hr. The binding capacity of LDL for ZnPc can be as high as about 60 phthalocyanine molecules per protein particle, although the final value is affected by the phospholipid composition of the liposomes and the liposome/lipoprotein ratio. HDL have a lower binding capacity (max. about 3 ZnPc/protein) as shown by studies of interlipoprotein transfer of the photosensitizer. The present findings indicate that the association of photosensitizers with lipid-based delivery systems, besides being necessary for water-insoluble compounds, affects their distribution among lipoproteins. Liposomes which are in a fluid state at the temperature of 37°C enhance the binding of ZnPc to LDL, which should increase the selectivity of tumour targeting by the phthalocyanine owing to the efficient receptor-mediated endocytosis of LDL by several types of malignant cells.

Keywords: Zinc-phthalocyanine Low-density lipoproteins High density lipoproteins Liposomes Photodynamic therapy

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INTRODUCTION

The plasma protein distribution of systemically injected tumour-photosensitizers depends on their physico-chemical properties (Kongshaug, 1992; Reddi, 1994) and controls their distribution among different compartments of the tumour tissue (Kessel *et al.*, 1987). In particular, hydrophilic photosensitizers are mainly transported in the bloodstream by albumin and localize in the vascular stroma of the tumour, while hydrophobic photosensitizers mainly bind to lipoproteins, including LDL, and localize preferentially in the neoplastic cells (Zhou *et al.*, 1988; Henderson and Bellnier, 1989) as well as in tumour-associated macrophages (Hamblin and Newman, 1994). In addition, it has been reported that the partitioning of the

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Abbreviations: BCA, bicinchoninic acid; DMPC, 1,2-dimiristoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; HDL, high-density lipoproteins; LDL, low-density lipoproteins; OOPS, 1,2-dioleoyl-sn-glycero-3-L-serine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; ZnPc, zinc(II)-phthalocyanine.

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photosensitizer among the plasma proteins influences the selectivity of tumour targeting (Jori and Reddi, 1993), although the overall weight and importance of this factor is still an open question (Korbelik, 1992; Kessel, 1992; Korbelik and Krosl, 1994). There is some evidence that the uptake of hydrophobic photosensitizers into neoplastic cells is favoured by the receptor-mediated endocytosis of LDL (Allison *et al.*, 1991; Maziére *et al.*, 1991). Fast replicating cells require large amounts of cholesterol for membrane synthesis and satisfy their cholesterol demand by enhancing the synthesis of endogenous cholesterol and/or the endocytosis of LDL (Gal *et al.*, 1981; Rudling *et al.*, 1990a). Thus many types of cancer cells express a higher number of LDL receptors than the corresponding normal cells (Rudling *et al.*, 1990b; Lombardi *et al.*, 1989). Based on these considerations, a higher and more selective uptake of LDL-associated antitumoural drugs is likely to occur (Peterson *et al.*, 1991). Several hydrophobic photosensitizers of interest in the photodynamic therapy of tumours are administered to tumour-bearing animals after incorporation into liposomes of various phospholid composition, Cremophor-EL emulsion or cyclodextrin inclusion complexes (Reddi *et al.*, 1990; Schieweck *et al.*, 1994; Woodburn *et al.*, 1994). Previous *in vitro* and *in vivo* studies with ZnPc and Sn(IV)-etiopurpurin suggest that the delivery vehicle can affect the distribution of the photosensitizer among the various lipoprotein families, hence their tumour uptake (Polo *et al.*, 1992; Garbo, 1990; Ginevra *et al.*, 1990; Kessel *et al.*, 1991; Kongshaug *et al.*, 1993). Most of the presently available information on the interaction of photosensitizers with the various lipoproteins have been obtained by delivering the photosensitizer to the whole plasma and measuring the relative percent distributions. On the contrary, very little is known on the interaction of photosensitizers with the individual lipoprotein classes, including the effect of the delivery system on this process. In this paper we report some studies on the binding of ZnPc with isolated LDL and HDL; i.e. the two lipoprotein classes responsible for the transport of more than 80% of hydrophobic photosensitizers circulating in the bloodstream. ZnPc was delivered to LDL or HDL after incorporation into small unilamellar liposomes of different phospholipid composition, so that the effect of different delivery systems could be evaluated.

MATERIALS AND METHODS

Chemicals

ZnPc and the liposomal formulation of ZnPc CGP 55847 were supplied by Ciba-Geigy (Basel, Switzerland) and used as received. The CGP 55847 formulation contained POPC and OOPS in a 9:1 weight ratio. In the same formulation the dye-to-lipid weight ratio was 1:100 (Schieweck *et al.*, 1994). DPPC and DMPC were purchased from Sigma Chemical Co. (St Louis, Mich., U.S.A.). The bicinchoninic acid (BCA) reagent for protein assay (Smith *et al.*, 1985) was supplied by Pierce (Rockford, Ill., U.S.A.). All solvents were analytical grade reagents and were used as received.

Animals

New Zealand rabbits (2.5 kg body wt) were supplied by Ditta Fasolato (Padova, Italy) and kept in standard cages with free access to tap water and standard dietary chow. The rabbits were fasted overnight before the experiments. Animal care was performed according to the guidelines established by the Italian Committee for Experiments on Animals.

Liposomes

ZnPc was incorporated into small unilamellar liposomes of DPPC or DMPC, in a 1:35 molar ratio to the phospholipids, by using the injection method of Kremer *et al.* (1977) modified as described by Ginevra *et al.* (1990). Vials of the liposomal formulation CGP 55847 containing colyophilized ZnPc (0.4 mg) and phospholipids (40 mg) were added with water (2 ml/vial), immediately before use, for liposome reconstitution (Isele *et al.*, 1994). The ZnPc concentration in the liposomal suspensions was determined by absorption spectroscopy using $\varepsilon = 2.41 \times 10^5\ M^{-1}\ cm^{-1}$ at 670 nm in pyridine.

Isolation of plasma lipoproteins

Blood collected from 3 to 4 rabbits was added with EDTA (1 mg/ml) and NaN_3 (0.1 mg/ml) and centrifuged at 3000 rpm for 10 min at room temperature to separate plasma from erythrocytes. The plasma from the different rabbits was pooled and the plasma protein classes, very low-density lipoproteins, LDL, HDL and heavy proteins (albumin + globulins) were separated by sequential ultracentrifugation (Havel *et al.*, 1955) using a TFT 70.38 rotor (Kontron Instruments) in an Ultra Centrikon T-2060 ultracentrifuge (Kontron Instruments). The LDL

(density (d) 1.006–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) fractions were dialyzed overnight against 500 ml of NaCl (11.42 g/l, d = 1.006 g/ml) with a change of the dialysing solution after 2 hr. The apo-protein concentration in the LDL and HDL fractions was determined using the BCA protein assay (Smith *et al.*, 1985). The lipoprotein concentration was obtained by using the apo-protein percentages for rabbit lipoproteins reported by Chapman *et al.* (1986). The lipoprotein concentrations indicated in the paper are referred to the holoprotein concentration.

Preparation of ZnPc–lipoprotein complexes

LDL or HDL were incubated at 37 C with ZnPc incorporated into DPPC, DMPC or POPC/OOPS liposomes for selected periods of time (1–6 hr). The LDL and HDL concentration in the incubation medium (NaCl 11.42 g/l) ranged between 1.01 and 2.01 mg/ml and 0.39 and 1.16 mg/ml, respectively. The ZnPc concentration ranged between 1 and 30 μg/ml. At the end of the incubation period the ZnPc–lipoprotein complexes were separated from liposomes by ultracentrifugation. This separation was achieved by using two methods (A and B) depending on the type of liposomes and lipoproteins studied. Method A is based on the ultracentrifugation of the liposome–lipoprotein mixtures at d = 1.063 and was utilized for the separation of DPPC liposomes from ZnPc-LDL complexes, as well as of POPC/OOPS liposomes from ZnPc–HDL complexes. Preliminary ultracentrifugation studies showed that at d = 1.063 the DPPC liposomes localize in the lower part of the ultracentrifuge tube while under the same conditions the POPC/OOPS liposomes float on the top. When method A was applied, 2 ml of the liposome–lipoprotein mixture were taken to d = 1.063 by the addition of 0.3972 ml of a KBr solution at d = 1.35 and placed in a centrifuge tube that was subsequently filled with a solution at d = 1.063. The tubes were placed in the TFT 70.38 rotor and centrifuged (36,300 rpm) for 24 hr at 4°C.

Method B is based on the ultracentrifugation of liposome–lipoprotein mixtures in a density gradient and was utilized for the separation of DPPC liposomes from ZnPc-HDL complexes, as well as of DMPC or POPC/OOPS liposomes from ZnPc–LDL complexes. In this case, 2 ml of the liposome–lipoprotein mixture were placed in an ultracentrifuge tube containing 0.228 g KBr and 0.05 g sucrose and carefully mixed. The sample, now with a background density of d = 1.10 g/ml, was sequentially overlayered with 3.1 ml of a solution at d = 1.10 g/ml (11.42 g/l NaCl, 113.48 g/l KBr), 4.7 ml of a solution at d = 1.06 g/ml (11.42 g/l NaCl, 75.98 g/l KBr) and 1.5 ml of distilled water. The tubes were centrifuged in a swinging-bucket rotor (SW 40.1, Beckman) for 24 hr at 39,000 rpm and 20°C. After centrifugation according to method A or B, the ZnPc–lipoprotein complexes were isolated after identification using a control tube containing LDL or HDL complexes stained with Sudan black before centrifugation (Terpstra *et al.*, 1981). The ZnPc content in each complex was determined by absorption spectroscopy measurements and the amount of ZnPc associated to LDL or HDL was expressed as mol ZnPc/mol lipoprotein. Under all experimental conditions we obtained a clear separation of the lipoprotein complexes in spite of the possible change in the density distribution of liposome-modified lipoproteins.

Transfer of ZnPc between LDL and HDL

ZnPc–LDL and ZnPc–HDL complexes obtained from DPPC liposomes were incubated for 2 hr at 37°C with native HDL and LDL, respectively. ZnPc–LDL complexes containing 6 or 10 ZnPc molecules/LDL particle were incubated with various HDL concentrations (0.01–1.16 mg/ml). The ZnPc–HDL complex containing an average of 1.6 mol ZnPc mol^{-1} HDL was incubated with LDL concentrations ranging between 0.013 and 0.19 mg/ml. After incubation, LDL and HDL (2 ml) were separated by ultracentrifugation at d = 1.063 g/ml and the amount of ZnPc bound to each lipoprotein class was determined by following the same procedure as described above.

RESULTS

Binding of ZnPc with LDL

ZnPc incorporated into DPPC liposomes (1 μg/ml) was gradually transferred to LDL (2.01 mg/ml) upon incubation at 37°C. The transfer of ZnPc to LDL as a function of the incubation time is shown in Fig. 1. Experiments performed under the same conditions but with ZnPc incorporated into POPC/OOPS or DMPC liposomes showed no effect of the incubation time on the amount of ZnPc transferred to LDL, (namely, 1.5 mol ZnPc mol^{-1} LDL) at least for time intervals between 1 and 6 hr. The moles of ZnPc bound/mol of LDL, after a

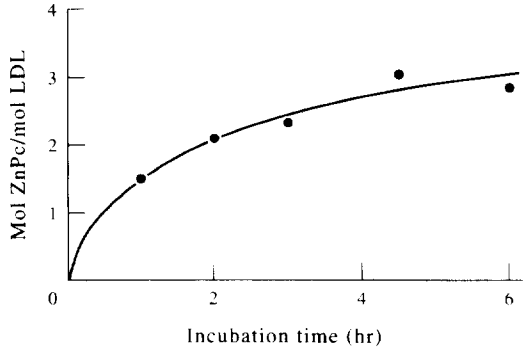

Fig. 1. Effect of the incubation time on the binding of DPPC liposome ZnPc delivered to isolated LDL. [ZnPc] = 1 μg/ml; [LDL] = 2.01 mg/ml. The values represent the average of several determinations with SD lower than $\pm$ 20%.

Table 1. Effect of the incubation time on the binding to HDL of DPPC liposome delivered ZnPc. The experiments were performed with different relative concentrations of ZnPc and HDL

Incubation time (hr)	Mol ZnPc/Mol HDL		
	A	B	C
1	0.2	1.3	1.7
2	0.3	1.5	2.5
3	0.3	1.6	2.9
4.5	0.3	1.7	2.9
6	0.3	1.8	3.3

A = ZnPc 1.0 μg/ml, HDL 1.15 mg/ml (molar ratio ZnPc/HDL 0.34).
B = ZnPc 6.5 μg/ml, HDL 1.15 mg/ml (molar ratio ZnPc/HDL 2.20).
C = ZnPc 6.5 μg/ml, HDL 0.39 mg/ml (molar ratio ZnPc/HDL 6.50).

given incubation time, depend on the initial ZnPc/LDL molar ratio in the incubation medium. When DPPC liposomes were used, the maximum number of ZnPc molecules bound to each LDL particle was 10–12 and was reached for an initial ZnPc/LDL molar ratio around 15 (see Fig. 2A). On the other hand, with the POPC/OOPS liposomes, the amount of ZnPc bound to LDL increased linearly at least up to an initial ZnPc/LDL ratio of 100 when about 50 ZnPc molecules were bound per LDL particle.

Binding of ZnPc with HDL

When DPPC liposomes were used as the delivery system, the binding of ZnPc with HDL was affected by the incubation time (Table 1). The effect was more evident at ZnPc/HDL molar ratios higher than 2. On the contrary, the amount of ZnPc delivered to HDL via POPC/OOPS liposomes was not affected by the duration of the incubation (1–3 hr) at all ZnPc/HDL molar ratios. For a fixed incubation time the number of ZnPc molecules bound to

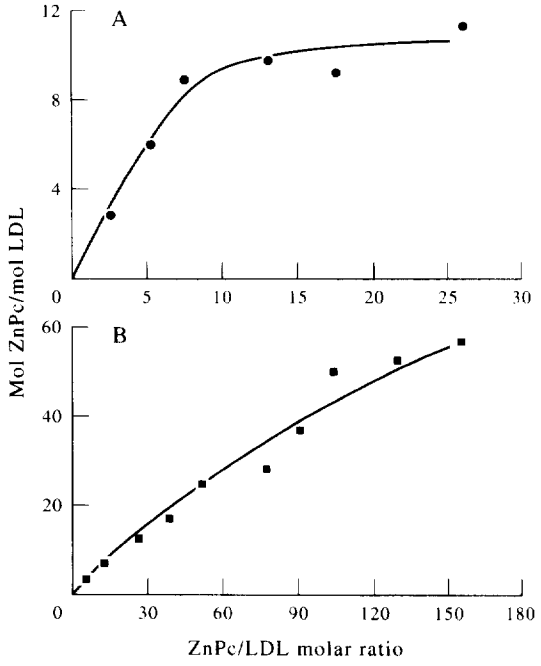

Fig. 2. Effect of ZnPc/LDL molar ratio on the binding of ZnPc to isolated LDL. ZnPc was delivered to LDL after incorporation into DPPC liposomes (part A) or into POPC/OOPS liposomes (part B). The values represent the average of several determinations with SD lower than $\pm$ 20%.

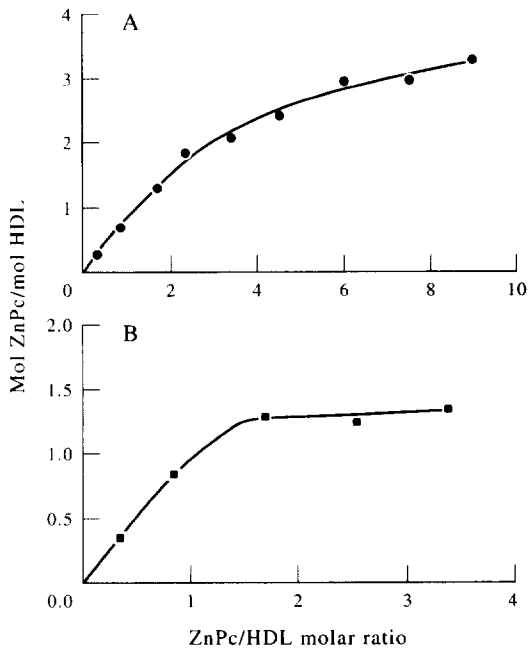

Fig. 3. Effect of ZnPc/HDL molar ratio on the binding of ZnPc to isolated HDL. ZnPc was delivered to HDL after incorporation into DPPC liposomes (part A) or into POPC/OOPS liposomes (part B). The values represent the average of several determinations with SD lower than $\pm$ 20%.

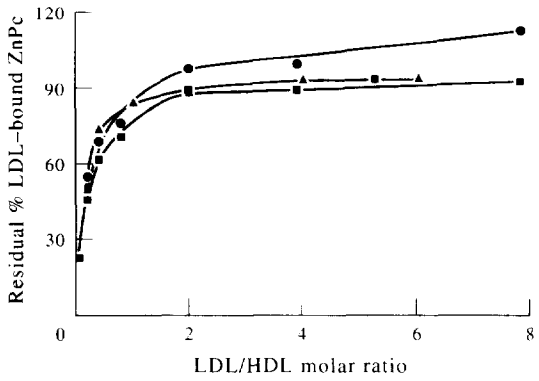

Fig. 4. Percentage of ZnPc associated to LDL as a function of the LDL/HDL molar ratio in the incubation medium. ZnPc–LDL complexes (1 mg/ml) containing 6 (■) or 10 ZnPc molecules/LDL particle (●) were incubated with HDL (0.01–1.16 mg/ml). ZnPc–HDL complexes (0.013–0.19 mg/ml) with an average ZnPc molecules of 1.6 were incubated in the same conditions with LDL (1 mg/ml) (▲). The values represent the average of several determinations with SD lower than ± 20%.

each HDL particle increased linearly with the ZnPc/HDL ratio in the incubation medium and tended to a plateau for ratios larger than 4 or 2 with the DPPC or POPC/OOPS liposomes, respectively (see Fig. 3). At high ZnPc/lipoprotein concentration ratios, DPPC liposomes induced a more efficient HDL binding of ZnPc than POPC/OOPS liposomes as one can see in the plots of Figs 3A and B.

Redistribution of ZnPc between LDL and HDL

ZnPc–LDL complexes containing 6 or 10 ZnPc molecules/LDL particle released the photosensitizer to HDL during incubation at 37 C. The percentage of ZnPc transferred to HDL was not affected by the number of ZnPc molecules initially bound to LDL; however, as one can see from Fig. 4 it was strongly affected by the relative concentrations of LDL and HDL. The percentage of ZnPc retained by LDL decreased as the LDL/HDL molar ratio decreased. For a LDL/HDL molar ratio equal to 1 about 75% of ZnPc was retained by LDL. The same final distribution of ZnPc between LDL and HDL was obtained when the photosensitizer was initially complexed with HDL and then added to photosensitizer-free LDL (see Fig. 4).

DISCUSSION

Previous studies demonstrated that ZnPc, incorporated into lipid-based delivery systems, is mainly bound to lipoproteins after incubation *in vitro* with whole plasma or after systemic administration to animals (Polo *et al.*, 1992; Ginevra *et al.*, 1990). Furthermore, the distribution of ZnPc among the different lipoprotein classes appears to be affected by the nature of the delivery vehicle. Since the formation of the photosensitizer–lipoprotein complexes may represent a crucial factor for the tumour uptake of the drug, it seems important to determine the parameters affecting the formation of such complexes. Toward this aim, the interaction of ZnPc with isolated LDL and HDL was studied with three ZnPc liposomal formulations. ZnPc in POPC/OOPS or DMPC liposomes is transferred very rapidly to LDL and HDL. The incubation time exerts no appreciable effect on the amount of ZnPc bound/lipoprotein particle at least for time intervals between 1 and 6 hr. That the association to lipoproteins of ZnPc in POPC/OOPS formulations follows a fast kinetics, was already reported for both isolated LDL and plasma (Versluis *et al.*, 1994; Rensen *et al.*, 1994). On the contrary, DPPC liposomes release ZnPc to LDL and HDL according to a slow kinetics that reaches a plateau after 5–6 hr incubation (see Fig. 1 and Table 1). It is likely that the different rate of ZnPc–lipoprotein association is determined by the different fluidity of the liposomes since at the incubation temperature of 37°C, the phospholipid bilayer of the POPC/OOPS and DMPC liposomes, is in a fluid state while that of the DPPC liposomes is in a quasi-solid state. Therefore, the incubation temperature, which modulates liposome fluidity, should play a major role in determining the rate of ZnPc binding with LDL or HDL, while the size of these liposomes (90–100 nm for POPC/OOPS, 30 nm for DMPC, 52 nm for DPPC) should be of minor importance. Any possible influence of the negative charge which is present in POPC/OOPS liposomes can be discarded since such liposomes show an identical behaviour with that typical of DMPC liposomes, which are electrically neutral (as DPPC). The amount of ZnPc bound to LDL and HDL depends on the initial ZnPc/lipoprotein molar ratio in the incubation medium (see Figs 2 and 3). In general, the number of bound ZnPc molecules per lipoprotein increases linearly up to an initial ZnPc/lipoprotein ratio that depends on the type of liposome and lipoprotein and then tends to a plateau. The binding of ZnPc to LDL reaches the plateau region for ZnPc/LDL molar ratios equal to 10 and 120 with DPPC and POPC/OOPS liposomes, respectively. As one can see from the plots in Figs 2A and B,

at high ZnPc/lipoprotein ratios, about 60 ZnPc molecules are associated to each LDL particle after delivery with the POPC/OOPS liposomes, while only 12 ZnPc molecules/LDL are bound after delivery with the DPPC liposomes. An efficient interaction between lipoproteins and ZnPc incorporated in POPC/OOPS liposomes, as well as the phospholipid components of these liposomes was recently reported by Versluis *et al.* (1994).

The transfer of ZnPc from POPC/OOPS and DPPC liposomes to HDL is a less efficient process as compared to LDL. As shown in Figs 3A and B, under saturating conditions, an average of 3 and 1 molecules of ZnPc are transferred to each HDL particle after incubation with DPPC and POPC/OOPS liposomes, respectively. The less efficient transfer of ZnPc to HDL can be explained, at least in part, by considering the size of the two lipoprotein particles (20–22 and 9–15 nm dia for LDL and HDL, respectively). In addition, a less efficient interaction of the liposomes with HDL as compared to LDL was hypothesized (Rensen *et al.*, 1994; Parks *et al.*, 1985). The molar plasma concentration of HDL is higher than that of LDL both in human and rabbit and this parameter can explain the high percentage ($\sim 70\%$) of ZnPc bound to HDL following incubation with whole plasma (Polo *et al.*, 1992).

Furthermore, the interlipoprotein transfer of ZnPc must be considered. As shown by the results reported in the present study, ZnPc bound to LDL or HDL can be transferred to lipoproteins free of ZnPc (see Fig. 4). The percentage of transferred ZnPc depends on the relative concentration of LDL and HDL in the mixture and for a given LDL/HDL molar ratio, the same final percent redistribution is observed whether ZnPc is initially bound to LDL or HDL. From the data reported in Fig. 4, one can see that for a LDL/HDL molar ratio equal to 1, about 75% of ZnPc is retained or tansferred to LDL. From these studies, it appears that LDL possesses an affinity for ZnPc higher than that of HDL. However, when the LDL and HDL concentrations in the incubation mixture approach those typical of the plasma, the percentage of ZnPc associated with LDL drops to about 25%, i.e. a value very similar to that found in the distribution studies with the whole plasma.

An overall examination of our data would also suggest that the dye/lipoprotein ratio in the initial complex may influence the interlipoprotein transfer efficiency. At low ZnPc/lipoprotein ratios the affinity of ZnPc for LDL or HDL is not appreciably affected, while differences in the efficiency are detected for higher ratios (Fig. 4). This could reflect a liposome-induced modification of the lipoprotein particle.

In conclusion, these studies suggest that unilamellar liposomes of different phospholipid composition release ZnPc to isolated LDL and HDL with a different rate and efficiency. In particular, POPC/OOPS liposomes release the incorporated ZnPc to LDL more efficiently than DPPC liposomes; the opposite is observed when the two types of liposomes are incubated with HDL (see Figs 2 and 3). It is important to mention that the incorporation of a large fraction of ZnPc and phospholipids into the LDL particle following incubation with POPC/OOPS liposomes leads to the production of a ZnPc–modified LDL complex that shows a diminished affinity toward an anti-lipoprotein B immunoglobulin (Versluis *et al.*, 1994). These observations suggest that the recognition of LDL by their specific receptor can also be decreased and, as a consequence, the internalization of the photosensitizer inside the tumour cells is less efficient. Another limiting factor of the tumour targeting by hydrophobic photosensitizer via the LDL-pathway is represented by the interlipoprotein transfer observed in the experiments reported here. Additional investigations are needed to elucidate the mechanism of photosensitizer transport *in vivo*.

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