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Interaction of Hydro- or Lipophilic Phthalocyanines with Cells of Different Metastatic Potential

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ABSTRACT. A highly metastatic (4R) and a nonmetastatic (RE4) transformed rat embryo fibroblast cell line were incubated with lipid-soluble Zn(II)-phthalocyanine (ZnPc) and its water-soluble tetrasulphonated derivative (ZnPcTS) and compared for phthalocyanine uptake. The hydrophobic liposome-delivered ZnPc showed a significantly greater uptake by both cell lines than did ZnPcTS. Moreover, the two phthalocyanines appear to interact with cells according to different pathways, as suggested by the different temperature-dependence of the binding process and the different inhibitory action exerted by selected serum proteins, such as lipoproteins and heavy proteins. Under all experimental conditions, the two cell lines exhibited similar interactions with ZnPc and ZnPcTS, suggesting that heterogeneity of the tumor cell population has a minor influence on the accumulation of photosensitizers. BIOCHEM PHARMACOL 51;5:585–590, 1996.

KEY WORDS. phthalocyanine; metastatic potential; serum proteins

It is well established that the tumor cell population is not homogeneous: even in the early stages of tumor growth, subpopulations of cells with different degrees of aggressiveness and varying metastatic potential are present within the primary tumor lesion [1]. This has been confirmed experimentally by the possibility of isolating subclones with different metastatic potential from human tumor cultures [2].

PDT† is a new technique for the treatment of neoplasias based on the ability of tumor tissues to retain some photosensitizers with a certain degree of selectivity; hence, photoactivation of the photosensitizer leads to tumor necrosis by the production of cytotoxic species [3]. It has been observed that the sensitivity of various tumors to PDT can be very different; moreover, a large variability has also been noted in the response of the same type of tumor to a given PDT protocol [4]. This behavior has been ascribed to various factors and tumor cell heterogeneity could be one element contributing to the lack of response to and failure of phototreatment.

At present, very little information is available regarding the influence of the cellular phenotype, such as malignant aggressiveness and metastatic potential, on the modalities and extent of photosensitizer uptake by cells as well as on their photosensitivity. This kind of information could be helpful for clinical applications, allowing one to tailor the PDT protocol to a given tumor type (e.g. by choosing the photosensitizer and the irradiation protocol that ensure the killing of those cells with the highest metastatic potential).

In this paper, we report on the uptake of phthalocyanines by two rat embryo fibroblast cell lines transfected with different oncogenes, RE4 and 4R. Both cells lines are tumorigenic but, while transfection with the *ras* oncogene makes 4R cells highly metastatic, cotransfection with *ras* and the pE1a plasmid confers tumorigenicity but not metastatic potential to RE4 cells [5]. Thus, these cells, cloned from a common parent cell and exhibiting a clear cut difference in their aggressive behavior, may represent a useful *in vitro* model to study the influence of the invasive phenotype on dye uptake.

We used two phthalocyanine-type photosensitizers, namely ZnPc and its tetrasulphonated derivative ZnPcTS. They are characterized by similar spectroscopic and physicochemical properties [6], but by different solubility because ZnPc is hydrophobic and lipid-soluble and ZnPcTS is water-soluble. ZnPc is now in phase I clinical trial for treatment of tumors of the aerodigestive tract.

MATERIALS AND METHODS

Cell Lines

Two rat embryo fibroblast cell lines were used: 4R, which is highly metastatic in nude mice, and RE4, which is only tu-

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† *Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FCS, foetal calf serum; OOPS, 1,2-dioleyl-sn-glycero-3-phospho-L-serine; PBS, phosphate-buffered saline; PDT, photodynamic therapy; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocoline; SDS, sodium dodecylsulphate; ZnPc, Zn(II)-phthalocyanine; ZnPcTS, tetrasulphonated Zn(II)-phthalocyanine.

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morigenic [7]. Briefly, 4R was derived by transfection into second passage rat embryo cells of the plasmid pEJ, which contains the activated form of the c-Ha-*ras* oncogene as cloned from the bladder carcinoma cell line T24. RE4 was derived by cotransfection of the plasmid pEJ and pE1a, which contains 2.5 kb of DNA sequences from the left end of the adenovirus type 2 genome. The two cell lines exhibit substantial differences in both their metastatic phenotype and basement membrane collagenolytic activity [5]. The cells were grown in DMEM (Gibco, Paisley, U.K.) containing 10% heat-inactivated FCS and supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin (Sigma, St. Louis, MO) and 2 mM glutamine (Sigma). The cell lines were routinely checked for absence of mycoplasm contamination.

Photosensitizers

ZnPc was added to the cells using liposomes as the delivery system. Small unilamellar vesicles of POPC (CGP 31 586) and OOPS (CGP 31 524A) with a ratio of 9:1 were supplied freeze-dried for storage by Ciba-Geigy (Basel, Switzerland) and rehydrated by adding water immediately before use. The photosensitizer:lipid molar ratio in the liposomes was 1:100 [8]. ZnPc concentration was usually 20 μg/mL.

ZnPcTS was a gift from J. E. van Lier (Sherbrooke, Canada) and was synthesized and purified according to Brasseur et al. [9]. A stock solution (1.5 mM) was prepared by dissolving the photosensitizer in water and storing at 4°C; the concentration was checked by absorption spectroscopy after dilution in methanol (ϵ at 668 nm is 1.8×10^5 M^{-1} cm^{-1} [9]). Before the experiments, a 20 μg/mL solution was prepared from the stock by dilution with water.

Serum Components

The lipoprotein and heavy protein fractions of fetal calf serum were prepared by ultracentrifugation following the procedure described by Rudel et al. [10]. Briefly, after raising the serum density to 1.225 g/mL by adding 0.3517 g of KBr per mL, the serum was centrifuged at 200,000 g_{av} for 40 hr with an SW 40 swinging bucket rotor (Beckman). According to this technique, lipoproteins (approximately 2 mL) concentrate at the top of the tube, and the bottom fraction contains the more dense serum proteins, that is, albumin and globulins. After fractionation, the proteins were dialyzed against 250 mL of saline (11.47 g/L of NaCl) for 24 hr with 2 changes of the dialyzing solution. Protein concentration was measured using the bicinchoninic acid assay [11]. The cholesterol content of the fractions was assayed using a kit purchased from Sentinel (Milano, Italy); in a typical separation, more than 85% of the serum cholesterol was recovered in the lipoprotein fraction.

Cellular Uptake of the Phthalocyanines

For the uptake experiments, cells (2×10^6) were seeded in 25 cm^2 flasks (Corning) and grown for about 20 hr in DMEM containing 10% FCS. Under these conditions, cells remained in the logarithmic phase of growth throughout the experiment. Liposomal ZnPc or aqueous ZnPcTS were diluted to the desired concentrations in DMEM and incubated with the cells after removal of the growth medium. The incubation was usually performed at 37°C in a dark humid atmosphere containing 5% CO_2; in some experiments incubation was performed for 2 hr at 4°C.

The effect of whole serum or serum protein fractions on phthalocyanine uptake was studied by adding the medium containing the photosensitizers with 10% of FCS or with different protein concentrations of the lipoprotein fraction or the heavy protein fraction immediately before incubation.

At the end of the incubation period, the medium containing the phthalocyanines was removed and the cell monolayer carefully washed twice with 4 mL of phosphate-buffered saline containing Ca^{++} and Mg^{++} ions (PBS, Flow, Irvine, Scotland). Then, 2 mL of a 2% aqueous dispersion of SDS were added to the flasks. After gentle magnetic stirring for 1 hr, each sample was divided into 2 portions: 0.5 mL was stored to assay the protein content, and 1 mL was immediately diluted in methanol in the case of ZnPcTS or in a mixture of methanol:chloroform (2:1) in the case of ZnPc and centrifuged at 2500 *g* for 15 min. The fluorescence emitted in the 630–800 nm spectral range upon excitation at 600 nm was measured for each sample with a Perkin Elmer MPF4 spectrophotofluorimeter; ZnPc or ZnPcTS concentrations in the samples were then calculated by interpolation with a calibration plot obtained by measuring the fluorescence emission intensity of solutions containing known photosensitizer concentrations.

The uptake of the phthalocyanines by the cells is expressed as nmoles of photosensitizer/mg of protein. During each experiment two flasks were seeded with the same number of cells and were used for cell counting during the experiments: a correspondence between the protein content of the flasks and the number of cells was calculated with an average of 218 ± 14 μg or 225 ± 40 μg of protein/10^6 cells for 4R and RE4, respectively.

RESULTS

The effect of ZnPc concentration on the uptake of the photosensitizer by 4R and RE4 cells after 2 hr incubation at 37°C, in serum-free medium is shown in Fig. 1. The uptake increases linearly with ZnPc concentration and a slight deviation from linearity is observed only for concentrations higher than 0.8 μM. The behavior of ZnPcTS is very similar to that of ZnPc (Fig. 2), but the uptake of this phthalocyanine by both cell types appears to be lower, especially at high dye concentrations. In general, no significant difference between the two cell lines is observed; one possible exception is the more efficient uptake of ZnPcTS by metastatic 4R as compared with less metastatic RE4 cells at low phthalocyanine doses. The amount of phthalocyanine accumulated by the 4R cells increases with the incubation time and reaches a plateau value after approximately 24 hr, as shown in Fig. 3. It appears that, at any incubation time, the amount of ZnPc accumulated by

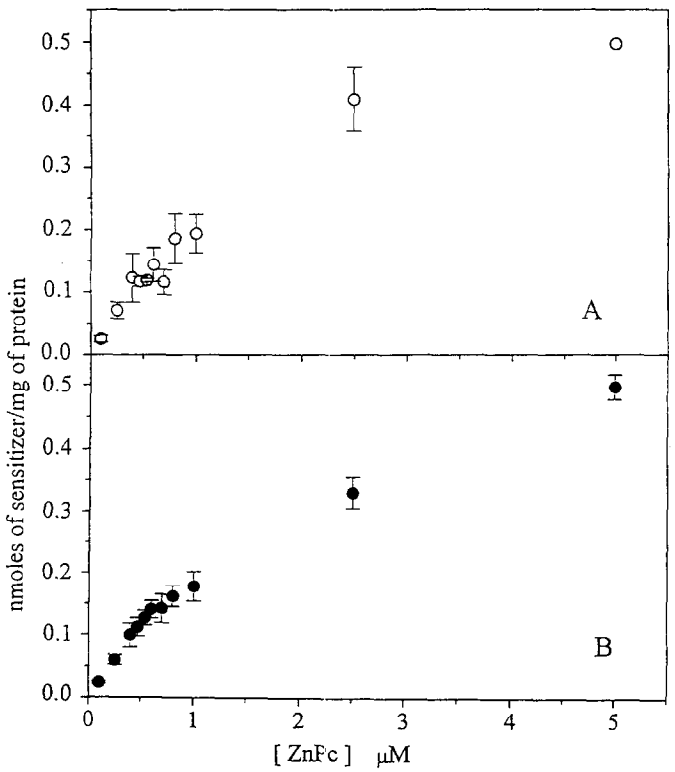

FIG. 1. Effect of phthalocyanine concentration on the uptake of ZnPc by 4R (A) and RE4 (B) cells. The incubation time was 2 hr.

the cells is higher than that of its sulphonated derivative. Figure 4 shows the kinetics of phthalocyanine uptake by RE4 cells: here, as well, liposomal ZnPc is accumulated in larger amounts. In both cell lines, the molar ratio between the amount of cell-bound ZnPc and ZnPcTS after 24 hr incubation is approximately 4.

To gain further information on the factors controlling the cellular uptake of the two photosensitizers, experiments were performed at the incubation temperature of 4°C. As shown in Table 1, the drop in temperature reduces the uptake of liposomal ZnPc by approximately 50% for both cell lines while; for

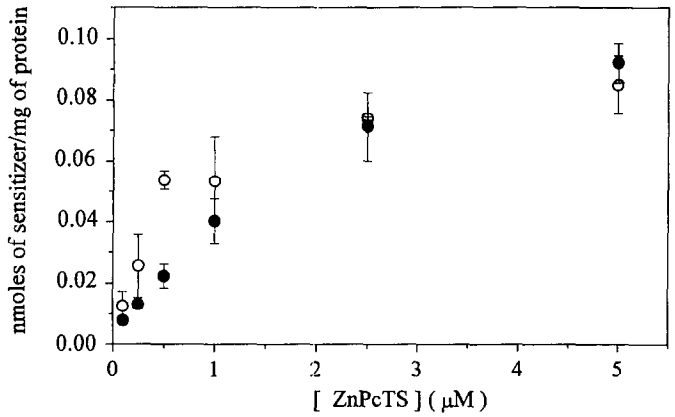

FIG. 2. Effect of phthalocyanine concentration on the uptake of ZnPcTS by 4R (○) and RE4 (●) cells. The incubation time was 2 hr.

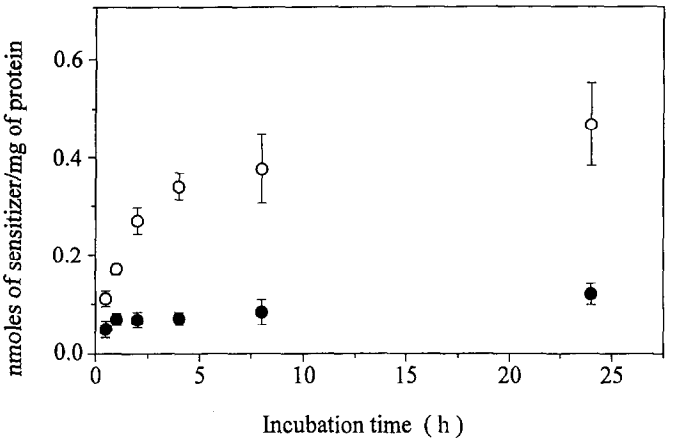

FIG. 3. Effect of the incubation time on the uptake of 2.5 μM ZnPc (○) and ZnPcTS (●) by 4R cells.

ZnPcTS, the temperature effect is less pronounced, although the amount of phthalocyanine accumulated at 4°C is lower than that accumulated at 37°C.

The addition of 10% serum, which corresponds to a total protein content of 3–3.4 mg/mL, reduces the uptake of both ZnPc and ZnPcTS by approximately 20–30% (Tables 2 and 3). To investigate whether or not the various serum proteins make different contributions to the inhibitory process, we performed separate cell incubations with the photosensitizer in the presence of either lipoprotein or heavy protein concentrations approximating those actually present in fetal calf serum; in particular, we found that lipoproteins represent ca. 2–3% of the overall protein content of the serum, in agreement with the findings of Korbelik [12].

Clearly, lipoproteins strongly inhibit the uptake of ZnPc by both 4R (Table 2) and RE4 cells (Table 3); for 0.07 mg/mL lipoprotein, equivalent to the lipoprotein concentration in the presence of 10% FCS (as based on cholesterol measurement), the observed inhibition is similar to that found with the serum. On the other hand, a limited inhibition of ZnPc uptake is caused by the addition of the heavy protein fraction (mainly albumin) even at protein concentrations higher than 3.4 mg/

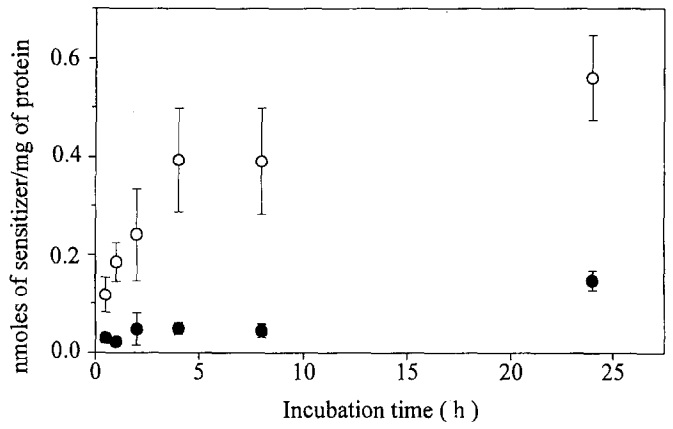

FIG. 4. Effect of the incubation time on the uptake of 2.5 μM ZnPc (○) and ZnPcTS (●) by RE4 cells.

TABLE 1. Effect of the incubation temperature on the uptake of 2.5 μM ZnPc and ZnPcTS by 4R and RE4 cells

Cell line	T (°C)	Recovery of ZnPc (ng/mg protein)	%	Recovery of ZnPcTS (ng/mg protein)	%
4R	37	188.8 ± 5.2	100	48.1 ± 12.2	100
	4	89.1 ± 4.3	47	36.1 ± 5.4	75
RE4	37	144.3 ± 28.3	100	32.1 ± 7.9	100
	4	75.7 ± 10.9	52	26.0 ± 3.1	81

The incubation of time was 2 hr.
Mean ± SD of 4 experiments.

mL, which corresponds to the heavy protein content in 10% FCS, and more than 20-fold higher than the lipoprotein concentration.

Very little, if any, inhibition of ZnPcTS uptake is caused by the addition of lipoproteins, whereas heavy proteins induce an inhibition that increases with increasing protein concentration.

Absorption spectroscopy studies have shown that 2.5 μM ZnPcTS is highly aggregated in PBS and that a partial monomerization can be obtained by adding 3.4 mg/mL of proteins from the heavy protein fraction; no effect is observed upon addition of 0.14 mg/mL of the lipoprotein fraction (data not shown).

DISCUSSION

Our results clearly show that both fibroblast cell lines studied in the present investigation, 4R and RE4, possess a higher affinity for the lipophilic ZnPc than for its water-soluble tetrasulphonated derivative ZnPcTS. Other authors have also found a good correlation between cellular uptake of porphyrin-type photosensitizers and their degree of hydrophobicity [13]. Thus, comparative studies on the uptake of tetraphenylporphine derivatives with various degrees of sulphonation by murine leukemia cells pointed out [14] that the efficiency of cell targeting decreases upon increasing the number of peripheral

TABLE 2. Effect of fetal calf serum (FCS) and serum protein fractions on the uptake of 2.5 μM ZnPc and ZnPcTS by 4R cells

Incubation medium	Protein conc. (mg/ml)	Percentage of uptake ZnPc	ZnPcTS
DMEM	–	100	100
DMEM + 10% FCS	3–3.3	80.9 ± 10.8	77.7 ± 17.1
DMEM + LIPOPROTEINS	0.07	88.7 ± 0.7	82.5 ± 6.8
	0.14	72.3 ± 4.1	105.9 ± 27.1
	0.28	58.1 ± 7.2	103.3 ± 20.8
DMEM + HEAVY SERUM PROTEINS	1.7	88.7 ± 6.3	96.2 ± 8.4
	3.4	83.8 ± 2.5	80.0 ± 17.2
	6.8	76.0 ± 0.4	67.9 ± 14.9

Incubation time was 2 hr. Recovery is expressed as percentage of Pc recovered from cells incubated in serum free medium.
Mean ± SD of 4 experiments.

TABLE 3. Effect of fetal calf serum (FCS) and serum protein fractions on the uptake of 2.5 μM ZnPc and ZnPcTS by RE4 cells

Incubation medium	Protein conc. (mg/ml)	Percentage of uptake ZnPc	ZnPcTS
DMEM	–	100	100
DMEM + 10% FCS	3–3.4	75.9 ± 5.3	72.4 ± 27.6
DMEM + LIPOPROTEINS	0.07	73.0 ± 11.3	79.8 ± 12.7
	0.14	49.0 ± 6.2	102.0 ± 9.4
	0.28	30.0 ± 10.2	88.9 ± 15.2
DMEM + HEAVY SERUM PROTEINS	1.7	91.7 ± 5.8	77.1 ± 21.1
	3.4	87.3 ± 2.0	83.9 ± 24.4
	6.8	89.3 ± 1.0	57.1 ± 10.0

Incubation time was 2 hr. Recovery is expressed as percentage of Pc recovered from cells incubated in serum free medium.
Mean ± SD of 4 experiments.

sulphonic substituents, with the tetrasulphonated compound displaying the lowest affinity.

The affinity of ZnPc for 4R and RE4 cells might be partially enhanced by the use of a liposomal delivery system. It was previously observed [15] that liposome-delivered porphyrins are accumulated by tumor cells in larger amounts than the same porphyrins dissolved in a homogeneous aqueous solution. Phospholipid vesicles can interact with mammalian cells through different modalities: in particular, liposomes (such as those used in the present work) that are in a fluid state throughout the temperature range studied by us (4–37°C) can either fuse with cells, undergo endocytosis, or exchange molecules with the cytoplasmic membrane [16]. An important involvement of endocytotic processes in the uptake of liposomal ZnPc by 4R and RE4 cells is suggested by the marked reduction in photosensitizer accumulation observed when the incubation temperature was lowered to 4°C; however, endocytosis of the phthalocyanine may not be the only mechanism involved, because a complete inhibition of this process should otherwise occur at 4°C [17]. It is likely that the uptake of sulphonated phthalocyanine involves different modes of interaction between the photosensitizer and the cell membrane, as suggested by the lesser sensitivity of this process to a decrease in temperature. Both endocytotic and passive diffusion mechanisms have been invoked for the uptake of tetrasulphonated porphyrins by tumor cells; the relative importance of the two pathways depends on the photosensitizer and the experimental conditions [18, 19]. Our findings would suggest that the passive diffusion mechanism is more active for ZnPcTS in the cell systems investigated.

The presence of serum or selected fractions of serum proteins also causes an inhibition of photosensitizer uptake. This has often been observed [12, 20, 21] and is generally interpreted as the result of a competition process between cellular binding sites and serum components for accumulation of photosensitizer molecules. In fact, the two phthalocyanines interact with different classes of serum proteins depending on their degree of hydrophobicity. Thus, liposome-bound ZnPc has been shown to be exclusively released to the lipoprotein frac-

tion [22]; consequently, it is reasonable that the addition of lipoproteins inhibits the cell uptake of this phthalocyanine, and very little effect is produced by the heavy proteins. On the contrary, the protein-bound fraction of both tetrasulphonated porphyrins [23] and phthalocyanines [24] is largely associated with heavy serum proteins. Again, it is, therefore, conceivable that cell uptake of ZnPcTS is inhibited to some extent by the latter protein fraction.

The observation that the two phthalocyanines interact with different serum proteins may have important consequences for determining the mechanism and efficiency of cell photosensitization. It has been reported [21] that both lipoproteins and heavy proteins, although interfering with cell accumulation of Zn-phthalocyanine, influence the subcellular distribution of the dye; hence, the nature of the photosensitive targets. This possibility is further supported by the fact that ZnPc and ZnPcTS are likely to be accumulated by cells according to different pathways, as indicated by the temperature dependency of their binding. Endocytosized photosensitizers are mainly associated with cell membranes, and dyes uptaken *via* passive diffusion predominantly localize in the cytoplasm and the nuclear region [18, 19]. Experiments are in progress to define the partitioning of ZnPc and ZnPcTS among the various cellular compartments.

Under all experimental conditions, the behavior of the two cell lines has been very similar. Corresponding conclusions were drawn by previous authors [25, 26], who observed no difference in the uptake of haematoporphyrin derivative by cells with different oncogenic potential. It has been suggested [26] that the accumulation of photosensitizers by different cells is correlated with the cell volume. However, our two cell lines have been found to have essentially identical dimensions [7]. Thus, the interaction between these cells and the photosensitizers seems to be modulated mainly by the characteristics of the photosensitizer and is not affected by the different metastatic potential shown by these two cell lines. This could be a relevant indication for clinical applications of PDT: heterogeneity of cell population in the tumor could be a secondary factor in determining the uptake of both lipophilic and hydrophilic photosensitizers and cell photosensitivity. Thus, the same PDT protocol could be utilized for the treatment of tumors having different metastatic potential.

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