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Zn(II)-phthalocyanines as phototherapeutic agents for cutaneous diseases. Photosensitization of fibroblasts and keratinocytes

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Abstract

Two tetrasubstituted (RLP024 and RLP040) and one monosubstituted (MRLP101) Zn-phthalocyanines were readily accumulated by three skin-derived cell lines (HT-1080 transformed human fibroblasts, 3T3 mouse embryo fibroblasts and HaCaT human keratinocytes) upon 1 h-incubation with 0.5–5 μM phthalocyanine concentrations. The affinity was markedly larger for the tetra- as compared with the mono-substituted phthalocyanine, even though smaller phthalocyanine amounts were generally recovered from keratinocytes. As a consequence, the two tetra-substituted phthalocyanines exhibited a higher phototoxicity against all the three cell lines. Typically, the cell survival decreased by at least 80% after 1 min irradiation with 600–700 nm light at a fluence-rate of 50 mW/cm^2 in the presence of 5 μM phthalocyanine.

Fluorescence microscopy and caspase-3 activation studies indicate that cell death of fibroblasts largely occurred by a random-necrotic process while the keratinocytes underwent cell death predominantly via apoptosis in spite of a very similar pattern of subcellular distribution of the phthalocyanines.

Keywords: Fibroblasts; Keratinocytes; Phthalocyanine; Photosensitization; Photodynamic therapy

1. Introduction

It is now well established that phthalocyanines represent a promising class of photodynamic sensitizers, which are especially suitable for phototherapeutic applications owing to their intense absorption bands in the far-red spectral region [1,2]. Light wavelengths in the 700 nm range are endowed with a deep penetration power into most human tissues [3]. In actual fact, the efficacy and potential of selected phthalocyanines are being investigated for the photodynamic therapy (PDT) of solid tumours [4], the photosterilization of blood [5], and the prevention of arterial restenosis by laser irradiation after balloon angioplasty [6]. In addition, cationic phthalocyanines appear to be efficient photosensitizing agents for the inactivation of a variety of microbial pathogens [7,8].

On the other hand, the use of phthalocyanines for the PDT of pathological conditions typical of cutaneous tissues has not been explored in detail so far, even though this phototherapeutic modality often gives positive results in the treatment of specific skin diseases, such as basal cell carcinomas, psoriasis and actinic keratosis [9]. In particular, the topical deposition of phthalocyanines on skin sites must address the problem posed by the relatively high molecular weight of the tetraazaisoindole derivatives, which hampers the crossing of the stratum corneum [10]. However, recent results from our laboratories (G. Roncucci, unpublished results) led to the development of a topical formulation of Zn(II)-phthalocyanines, which allows

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the penetration of sufficiently large amounts of photosensitizer into the epidermal layers. As a consequence, an extensive photoresponse is induced upon visible light irradiation of the phthalocyanine-loaded area. On the basis of these observations, we decided to undertake a systematic study on the efficiency of newly synthesized Zn-phthalocyanines (Pcs) as PDT agents for skin diseases. As a first step in this direction, we investigated the photosensitizing activity of selected phthalocyanines towards two cell lines, such as fibroblasts and keratinocytes, which are among the most important constituents of cutaneous tissues.

2. Materials and methods

2.1. Chemicals

The photosensitizers, 2,9(10),16(17),23(24)-Tetrakis-(4-oxy-*N*-methylpiperidinyl) phthalocyaninate zinc(II) (RLP024) [11], 2,9(10),16(17),23(24)-Tetrakis[3-(*N*,*N*,*N*-trimethylammonium)phenoxy]phthalocyaninate zinc(II) iodide (RLP040) and 2-[3-(*N*,*N*-dimethylamino)phenoxy]phthalocyaninate zinc(II) (MRLP101), were prepared by chemical synthesis and chemically characterized in the laboratories of Molteni Pharmaceuticals (Florence, Italy). The concentration of the phthalocyanines was determined spectrophotometrically in dimethylformamide (DMF). A molar extinction coefficient of 75,210 M^{-1} cm^{-1} at 681 nm for RLP024; 160,700 M^{-1} cm^{-1} at 678 nm for RLP040, and 200,570 M^{-1} cm^{-1} at 672 nm for MRLP101 were used.

The chemical structure of the phthalocyanines is shown in Fig. 1.

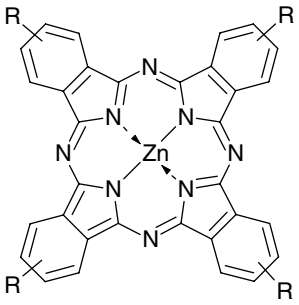
Sodium dodecylsulphate (SDS) was purchased from Merck. All other chemicals and solvents used were commercially available products of at least analytical grade.

2.2. Cell lines

Three cell lines were used as model constituents of skin tissues, namely: HT-1080, transformed human fibroblasts [12]; HaCaT, spontaneously immortalized human keratinocytes [13]; and Balb/3T3, mouse embryo fibroblasts [14]. The cells cultured as a monolayer at 37 °C in a humidified atmosphere with 5% CO_2 were grown in Dulbecco's modified minimal essential medium (DMEM) (Sigma) containing 10% heat-inactivated foetal calf serum (FCS) (Gibco) and supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin and 2 mM glutamine (Sigma).

2.3. Cellular uptake of the phthalocyanines

For the uptake experiments, cells ($4–5 \times 10^5$) were seeded in 25 cm^2 flasks (Falcon) and grown for about 24 h in DMEM containing 10% FCS. Stock solutions of phthalocyanines in PBS were diluted in DMEM to the desired concentrations (0.5–5 μM) and incubated with the cells after removal of the growth medium. The incubation



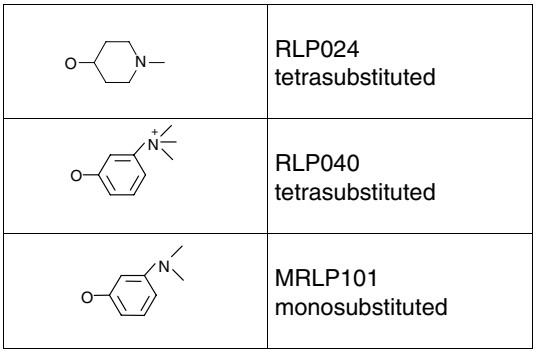


Fig. 1. Chemical structure of the phthalocyanines used in the present investigation.

was usually performed for 1 h and at 37 °C in a dark humid atmosphere containing 5% CO_2. At the end of the incubation period, the medium containing the phthalocyanines was removed and the cell monolayer was washed twice with 4 mL of phosphate-buffered saline (PBS) containing Ca^{++} and Mg^{++} ions. Then, 2 ml of a 2% aqueous dispersion of SDS were added to the flasks. After gentle magnetic stirring for 1 h, each sample was divided into 2 portions: 0.5 ml was stored to assay the protein content by the test with bicinchoninic acid [15], and 1 ml was immediately diluted with 2% SDS. The fluorescence emitted in the 650–800 nm interval was measured for each sample with a Perkin–Elmer MPF4 spectrophotofluorimeter. The phthalocyanine concentration in the samples was 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 was 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: a correspondence between the protein content of the flasks and the cell number was calculated with an average of 255 ± 75 μg of protein/10^6 HT-1080 cells, 183 ± 20 μg of protein/10^6 HaCaT cells and 150 ± 29 μg of protein/10^6 Balb/3T3 cells.

2.4. Cell photosensitization studies

For the photosensitization experiments, cells were seeded in Petri dishes at a density of 5×10^4 cells/cm^2

and grown for about 20 h in DMEM containing 10% FCS. Stock solutions of Pcs in PBS were diluted to the desired concentration in DMEM and incubated with the cells at 37 °C after the removal of the growth medium. After 1 h incubation, the cells were rinsed twice with PBS containing Ca^{++} and Mg^{++} ions and irradiated for 1 min in the same buffer by means of a Waldmann halogen lamp (Schwenningen, Germany) which was equipped with a set of band-pass optical filters to isolate the 600–700 nm spectral interval. The fluence rate at the irradiation site was 50 mW/cm^2.

The cell survival was measured by means of the trypan blue exclusion test 18–24 h after the end of irradiation.

2.5. Caspase-3 activity

The activity of caspase-3 was measured in the photosensitized cells at various post-irradiation times. The ApoAlert CPP32 kit (Clontech, Palo Alto, CA) was used. In a typical experiment 10^6 cells were collected by centrifugation, resuspended in 50 μl of lysis buffer, and held for 10 min on ice. Then, 50 ml of the reaction buffer containing DTT (dithiothreitol) and 5 μl of DEVD-AFC (asp-glu-val-asp-7-amino-4-methyl-coumarin) were added to the cell lysate, and after 1 h incubation at 37 °C the fluorescence emitted at 505 nm ($\lambda_{ex} = 400$ nm) was measured with a Perkin–Elmer LS50 spectrophotofluorimeter. The caspase-3 activity in the treated cells was expressed as a *n*-fold increase of the emitted fluorescence, taking the fluorescence from untreated cells as a reference.

2.6. Nuclear fragmentation

At 6 h after the end of the treatment, photosensitized and control cells were rinsed twice with PBS and incubated in the dark for 10 min with PBS containing Höechst dye HO33342 (HO342) at a concentration of 5 μg/ml.

Nuclear fragmentation was assessed by fluorescence microscopy (Zeiss, Germany). The excitation wavelength was 353–377 nm with emission monitored at 420–450 nm.

2.7. Fluorescence microscopy

The intracellular localization of RLP024 after 1 h incubation with a 10 μM phthalocyanine concentration was determined by an Olympus IMT-2 fluorescence microscope equipped with a refrigerated CCD camera (Micromax, Princeton Instruments). A 75-W Xenon lamp was used as the excitation source. Fluorescence images obtained with a 60×1.4 NA oil immersion objective (Olympus) were acquired and analysed with the imaging software Metamorph (Universal Imaging). Appropriate excitation (610 nm) and emission (670 nm) cubes (Chroma Technology Corp.) were used.

3. Results

3.1. Cell photosensitization studies

All the phthalocyanines selected for the present investigation were accumulated by fibroblasts and keratinocytes in amounts which steadily increased with increasing photosensitizer concentration in the incubation medium. As shown in Fig. 2, no apparent saturation of phthalocyanine binding by the three cell types was observed up to a 5 μM phthalocyanine concentration. It is worth underlining that the recoveries reported in Fig. 2 have been measured after two cell-washing steps, hence they represent tightly bound phthalocyanines. The extent of phthalocyanine accumulation appears to be markedly influenced by both the chemical structure of the photosensitizer and the cell type. Thus

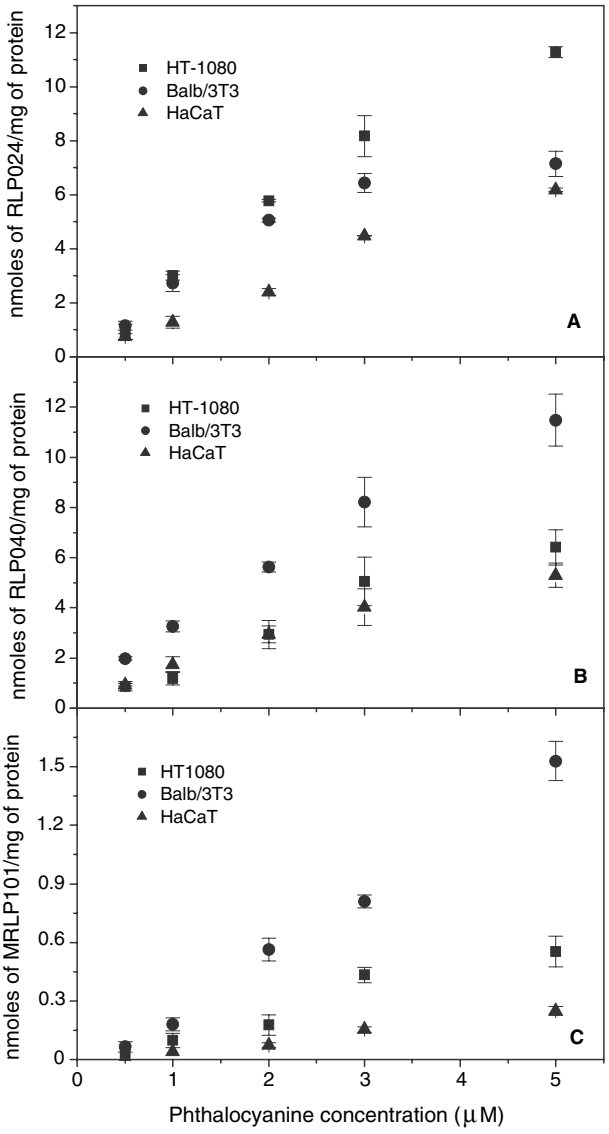


Fig. 2. Effect of the phthalocyanine concentration on the uptake of RLP024 (A), RLP040 (B) and MRLP101 (C) by different cell lines after 1 h incubation. Values represent means ± SD of at least three separate experiments.

a comparative analysis of the three panels in Fig. 2 suggests the following conclusions:

(a) Both tetrasubstituted phthalocyanines display a significantly higher affinity for the three cell types than the monosubstituted MRLP101 derivative: actually, 8–10-fold larger amounts of RLP024 and RLP040 are recovered from the cell samples after 1 h incubation for all the phthalocyanine concentrations used by us. This finding is somewhat surprising: mono-substituted phthalocyanines such as MRLP101 are more hydrophobic than phthalocyanines bearing four relatively polar substituents, and hydrophobic porphyrin derivatives are generally taken up in particularly large amounts by neoplastic or rapidly proliferating cells [16].

(b) The tetra-(*N*-methyl-piperidinyl) phthalocyanine (RLP024) and its tetra-(*N*,*N*,*N*-trimethyl-anilinium) analogue (RLP040) show an essentially identical affinity towards keratinocytes. On the other hand, some specificity seems to exist as regards the interaction of these phthalocyanines with fibroblasts, since RLP040 binds to 3T3 mouse fibroblasts in appreciably larger amounts as compared with HT-1080 human fibroblasts, while the opposite situation is observed in the case of RLP024. Thus, it is likely that the uptake of phthalocyanines by 3T3 and HT-1080 fibroblasts is modulated by subtle differences in the interaction between the tetraazaisoindole derivatives and the three-dimensional architecture of specific sub-cellular domains. In our case, such differences may by enhanced by the cationic character of RLP040 as compared with the neutral RLP024 molecule. Further studies to obtain more detailed information on this issue are in progress.

(c) The keratinocytes clearly bind substantially smaller amounts of the three phthalocyanines as compared with mouse and human fibroblasts. A preferential labelling of fibroblasts with respect to other constituents of mouse skin (including keratinocytes) has been reported also for other porphyrin-type photosensitizers, such as porphycenes [2].

In all cases, no detectable decrease in the survival of fibroblasts and keratinocytes was caused by incubation of the cells in the dark with the largest phthalocyanine concentrations investigated. Therefore, it is unlikely that important cytotoxic effects are induced under our experimental conditions in the absence of light. On the other hand, exposure of the phthalocyanine-loaded cells to red light wavelengths resulted in a drop of cell survival, as shown in Fig. 3. As one might have expected on the basis of the phthalocyanine binding studies, the photosensitivity of fibroblasts and keratinocytes (Fig. 3A and B) in the presence of RLP024 and RLP040 is significantly larger than that induced by MRLP101 (Fig. 3C). In particular, both the tetrasubstituted phthalocyanines yielded an essentially complete cell lethality after 1 min-irradiation with a 3 μM photosensitizer concentration. Interestingly, for a given phthalocyanine, the three cell types underwent closely similar degrees of photoinactivation in spite of 3–6-fold differences in the amount of cell-bound dye. Various hypotheses can be proposed in order explain this finding, such as (a)

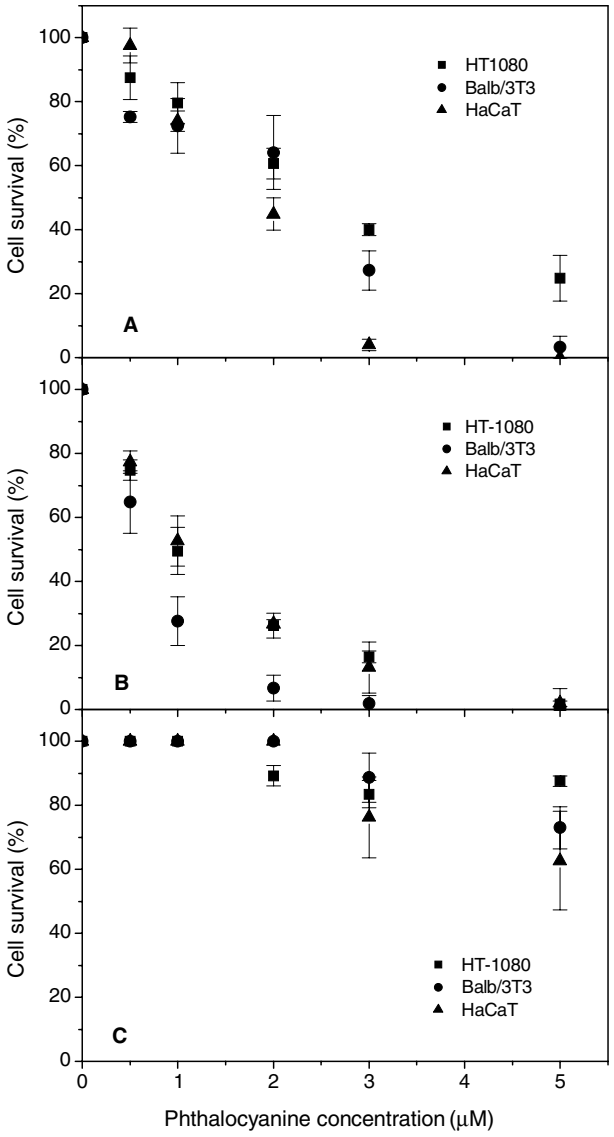


Fig. 3. Effect of the phthalocyanine concentration on the survival of different cell lines incubated for 1 h with RLP024 (A), RLP040 (B) and MRLP101 (C) and irradiated for 60 s with red light (600–700 nm) at a fluence rate of 50 mW/cm^2. Values represent means ± SD of at least three separate experiments.

keratinocytes, which appear to accumulate lower amounts of phthalocyanine, possess an intrinsically higher susceptibility to photooxidative attack than fibroblasts; or (b) the photosensitivity of the cells reaches a maximum level upon incubation with relatively small phthalocyanine concentrations.

3.2. Studies on the mechanism of cell photosensitization

The studies on the mechanism involved in cell photoinactivation were performed by using RLP024 which displayed the highest photosensitizing efficiency.

As a first step, we determined the expression of caspase-3, a typical marker of apoptotic processes [17], in photosensitized cells as a function of post-irradiation time. A variety

of reports pointed out [18] that the cell death via apoptosis often occurs as a consequence of photodynamic reaction pathways. The activation of caspase-3 provides a reliable index for apoptosis since it usually takes place in the final steps of the cascade of events typical of such modality of cell death [19]. As shown in Fig. 4 a very limited development of apoptosis was noticed in untreated cells. The appearance of apoptosis was clearly stimulated by photosensitization with RLP024 and maximal expression of caspase-3 could be measured at about 6 h after the end of the phototreatment. In particular, the activation of this enzyme occurred at a faster rate and in greater amounts in the case of keratinocytes. Thus, these cells appear to be appreciably more prone to undergo photoinduced apoptosis than fibroblasts, at least with the RLP024 phthalocyanine as a photosensitizer. Further support to this conclusion was lent by fluorescence microscopic analyses after staining of the photosensitized cells with a nuclear marker such as Höechst 342. The micrographs obtained for the three cell types (Fig. 5) indicate that control untreated cells (left panel) showed blue-fluorescent nuclei of normal shape with a slightly granular texture. On the other hand, the HO-342-labelled nuclei of photosensitized HaCaT keratinocytes exhibited (Fig. 5f) a clearly detectable chromatin condensation and fragmentation pattern which are typical of apoptosis. No evidence of apoptosis was shown by the nuclei of photosensitized HT-1080 and 3T3 fibroblasts, as one can see in Fig. 5b and c.

The different types of photoresponse observed for keratinocytes and fibroblasts is unlikely to be the consequence of major differences in the sub-cellular distribution of RLP024. Fig. 6 shows the fluorescence micrographs obtained for the various cell types after 1 h incubation with the phthalocyanine; the images obtained with the same samples upon observation in a bright field are placed in the right panel of Fig. 6. In all cases, the RLP024 fluorescence is mostly localized in correspondence of the cytoplasmic membrane. No broadening or redistribution of the emitted fluorescence was induced by photosensitization with the RLP024 phthalocyanine.

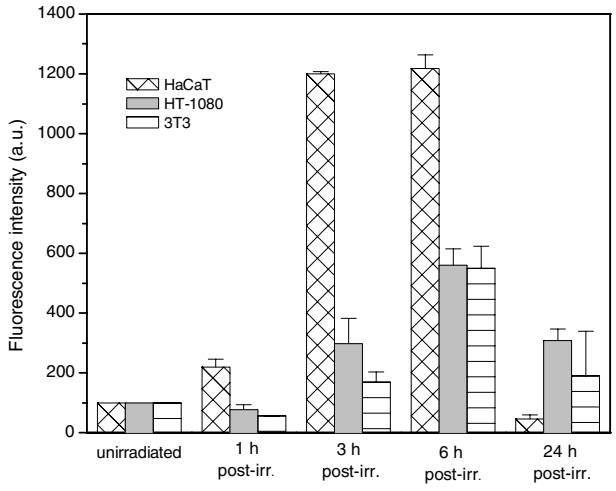


Fig. 4. Activation of caspase-3 in different cell lines at various time periods after PDT treatment with RLP024. Values represent means ± SD of at least three separate experiments.

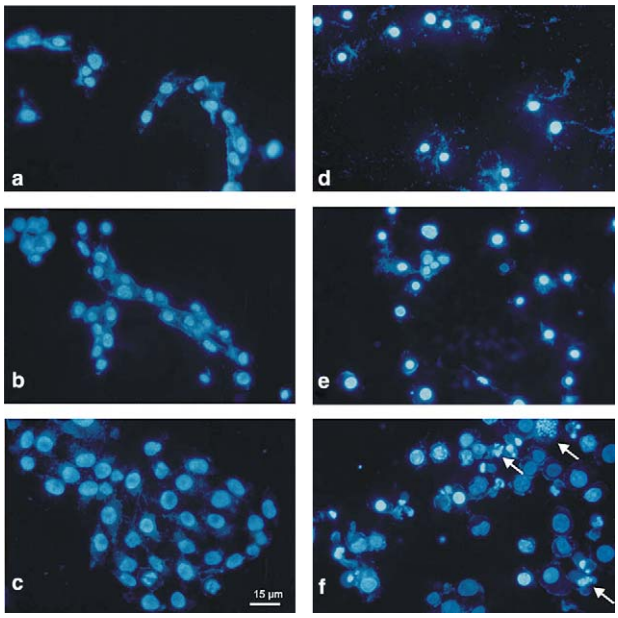


Fig. 5. Fluorescence micrographs of cells stained with HO342 at 6 h after phototreatment (1 h incubation with 5 μM RLP024, 60 s irradiation at 50 mW/cm^2): (a,b) Balb/3T3; (c,d) HT-1080 and (e,f) HaCaT. The HO342-labelled nuclei of HaCaT keratinocytes show the chromatin condensation and fragmentation typical of apoptosis. In contrast, nuclei of HT-1080 and Balb/3T3 fibroblasts show no evidence of apoptosis. Control cells (LEFT PANEL) show blue fluorescent nuclei with a slightly granular texture.

4. Discussion

One interesting finding of the present investigations is certainly represented by the observed larger affinity of relatively polar tetrasubstituted phthalocyanines for the three different types of cells as compared with a monosubstituted hydrophobic phthalocyanine derivative. Previous observations from several laboratories [20] consistently agree on the fact that lipophilic porphyrins and porphyrin analogues (including phthalocyanines) are accumulated in especially larger amounts by a variety of transformed cells; this finding is at the basis of the PDT of tumours [21]. In our case, HT-1080 fibroblasts are tumorigenic cells with a high mitotic index; however, an identical situation appears to occur for non-transformed 3T3 fibroblasts and HaCaT keratinocytes. Therefore, the peculiar behaviour observed in the present studies might be ascribed to a specific effect brought about by the piperidine- and aniline-type peripheral substituents which are present in our phthalocyanines, and whose influence on the photophysical and photobiological properties of porphyrinoid compounds has not been investigated so far, at least to our knowledge. This hypothesis can be ascertained only through an extension of our research to other cell systems, which is presently in progress in our laboratories.

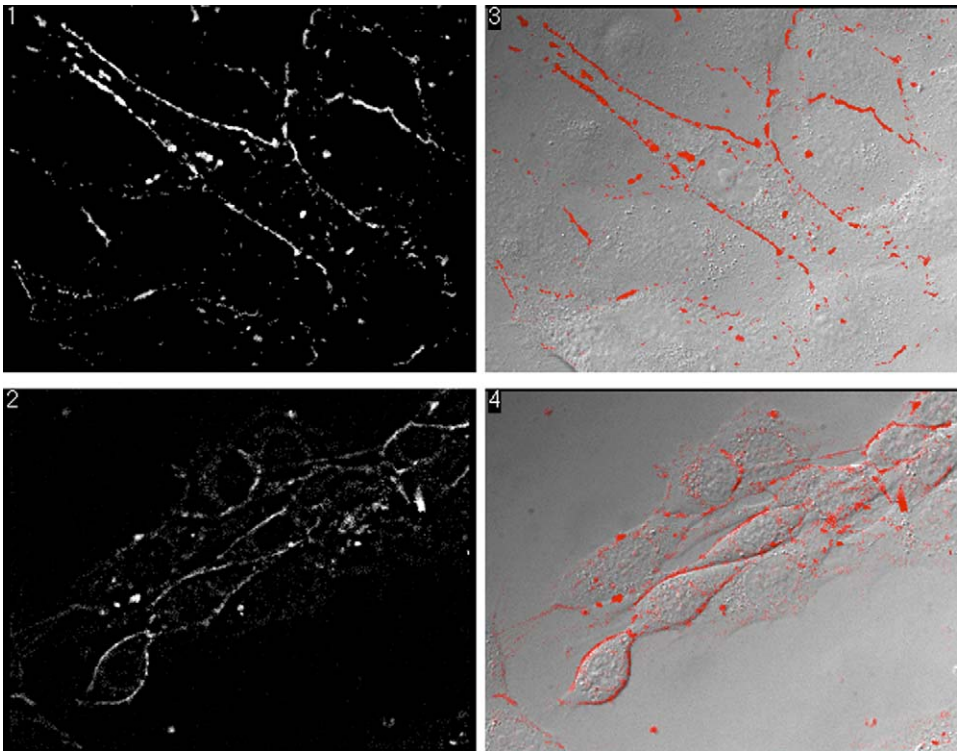


Fig. 6. Fluorescence micrographs of cells after 1 h incubation with 10 μM RLP024 showing the phthalocyanine fluorescence mostly localized at the level of the cytoplasmic membrane. The three cell lines exhibit a similar distribution (excitation at 616 nm and fluorescence detection between 650 and 700 nm): (1,2) HT-1080 and (3,4) HaCaT. Left panel: fluorescence images; right panel: overlayed bright field and fluorescence images.

It is also apparent that both RLP024 and RLP040 are very efficient photosensitizers of fibroblasts and keratinocytes, since an extensive and irreversible inactivation of such cells is achieved by using mild irradiation protocols. Now, fibroblasts and keratinocytes are among the most abundant constituents of cutaneous tissues; as a consequence, these two phthalocyanines appear to represent promising candidates as phototherapeutic agents for skin diseases, especially for those pathologies which can be treated by topical application of the photosensitizer.

Lastly, it appears that under our experimental conditions fibroblasts and keratinocytes undergo photosensitized inactivation at a closely similar rate and to an essentially identical extent in spite of the largely different mechanisms which are responsible for cell death. This is predominantly caused by apoptotic pathways in keratinocytes and most probably through random necrosis in fibroblasts. The reasons for such differences are not obvious since RLP024 shows an apparently very similar subcellular distribution pattern in HT-1080 and HaCaT cells: its main binding sites are localized at the level of the cytoplasmic membrane. It is well known [19] that the competition between apoptotic and necrotic processes leading to eventual cell death is often controlled by a delicate balance between a variety of experimental variables. In particular, one pathway to the photosensitized induction of cell apoptosis involves a modification in the microenvironment of phophatidylserine and the consequent activation of membrane-associated phospholipases [18] as suggested by the fluorescence microscopic data. Thus, it is possible that subtle differences exist between the microenvironments of RLP024 in the membranous districts of HT-1080 and HaCaT, thereby originating different subcellular responses.

In any case, our results would suggest that apoptosis and random necrosis are equally efficient in promoting photosensitized cell death, at least in the specific experimental systems studied in the present investigation. Thus, it would be of great interest to define the conditions which enhance the probability of apoptotic-type damage of cutaneous tissues: in actual fact, this kind of damage is not expected to cause the development of inflammatory processes [21], so that this therapeutic modality could be associated with better qualitative results (e.g. from the cosmetic point of view) and less pain for the patient. Moreover, and most important, the enhancement of the importance of apoptotic pathways during phthalocyanine photosensitization could be a key for orientating the overall photoprocess toward keratinocytes, which would minimize the risk of photoinduced damage to fibroblasts; this feature is particularly important if this technique has to be applied for the treatment of specific pathologies, such as non-melanoma skin cancer.

5. Abbreviations

DMEM Dulbecco's modified minimal essential medium
DMF dimethylformamide
FCS foetal calf serum

HO342 Höechst dye HO33342
Pcs phthalocyanines
PDT photodynamic therapy

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