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Phototoxicity of Protoporphyrin IX, Diarginine Diprotoporphyrinate and *N,N*-Diphenylalanyl Protoporphyrin Toward Human Fibroblasts and Keratinocytes *In Vitro*: Effect of 5-Methoxypsoralen¶

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ABSTRACT

The phototoxicity of two new porphyrin photosensitizers, diarginine diprotoporphyrinate ($PP(Arg)_2$) and *N,N*-diphenylalanyl protoporphyrin ($PP(Phe)_2$), and the synergistic effect of 5-methoxyposralen (5-MOP) have been studied in comparison with that of protoporphyrin IX (PPIX). Under ultraviolet-A (UV-A) irradiation ($\lambda = 365$ nm), the phototoxicity of the porphyrins toward cultured human fibroblasts and keratinocytes decreases in the order: PPIX > $PP(Arg)_2$ > $PP(Phe)_2$. A synergistic effect of 5-MOP on the phototoxicity of PPIX, $PP(Arg)_2$ and $PP(Phe)_2$ has been observed. The combination of PPIX, $PP(Arg)_2$ and $PP(Phe)_2$ with 0.1–0.5 μ*M* 5-MOP significantly potentiates the phototoxicity of the three porphyrins. The most effective potentiation was observed with the water-soluble $PP(Arg)_2$ and 5-MOP concentrations lower than 0.75 μ*M*. Above this 5-MOP concentration this potentiation is abolished. The intracellular concentration of PPIX and $PP(Phe)_2$ is independent of the presence of 5-MOP. On the other hand, the intracellular content of $PP(Arg)_2$ is decreased in a concentration-dependent manner by the psoralen. Illumination with red light, not absorbed by 5-MOP, leads to a weak potentiation of the $PP(Arg)_2$ phototoxic effect in the presence of 5-MOP, suggesting that dark interaction of 5-MOP with cell membranes aggravated by porphyrin photosensitization is involved in the observed phenomena. The results are tentatively explained by differences in hydrophobicity and molecular structures of the examined photosensitizers. PPIX, which is barely soluble in water, has a significantly higher affinity for cell membranes and simultaneously exerts a stronger phototoxic effect than $PP(Arg)_2$ whose solubility in water is high. On the other hand, the weak phototoxicity of $PP(Phe)_2$ could be explained by the steric hindrance brought by the phenylalanyl substituents on the pyrrole ring. The loss in the $PP(Arg)_2$ cell content probably explains the inhibition of the synergistic effect of 5-MOP on the $PP(Arg)_2$ phototoxicity at high 5-MOP concentration. This study suggests that $PP(Arg)_2$ in combination with 5-MOP might reveal a strong phototoxic effect when applied to skin cancer treatment.

INTRODUCTION

Photodynamic therapy (PDT) is an evolving modality of photochemotherapy applied in the treatment of various benign and malignant hyperproliferative diseases (1). In dermatology this method is an alternative treatment of benign proliferation (psoriasis), precancerous lesions (actinic keratosis) and several types of cancer, such as Bowen's disease, squamous cell carcinoma and basal cell carcinoma (2,3).

The PDT action is based on photoactivation of sensitizers selectively localized in proliferating cells, producing reactive oxygen intermediates (mainly singlet oxygen) that cause lethal damage to tissue or inhibit angiogenesis (or both). The most commonly used photosensitizers are porphyrin derivatives, such as porfimer sodium (Photofrin) first approved by U.S. Food and Drug Administration. However, this mixture of photosensitizers has many drawbacks. Besides its complex formulation and chemical heterogeneity, it has a very small absorption coefficient in the red region. Furthermore, its selectivity toward proliferating cells is relative—so that it is also retained in adjacent disease-free tissues–and its clearance from skin is very slow. Thus, strong skin phototoxic reactions have been noticed (4).

For these reasons, new strategies for improving the efficacy of PDT are needed. Two major options are possible. First, the synthesis of well-defined photosensitizers with less skin retention time and absorbing light of longer wavelengths than Photofrin has led to the so-called second-generation PDT photosensitizers (5–7). Another strategy is the development of combination therapies associating PDT with another treatment allowing the use of lower photosensitizer or light doses (or both). In this regard, it was

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Abbreviations: DMEM, Dulbecco's modified minimum essential medium; EMEM, Eagle's minimum essential medium with Earle's salts; FCS, fetal calf serum; HBSS, Hank's buffered saline solution; ID_{50}, dose inhibiting 50% of membrane integrity; 5-MOP, 5-methoxypsoralen; NR, neutral red; PBS, phosphate-buffered saline solution; PDT, photodynamic therapy; $PP(Arg)_2$, diarginine diprotoporphyrinate; PPIX, protoporphyrin IX; $PP(Phe)_2$, *N,N*-diphenylalanyl protoporphyrin; PUVA, psoralens + ultraviolet-A; UV-A, ultraviolet-A.

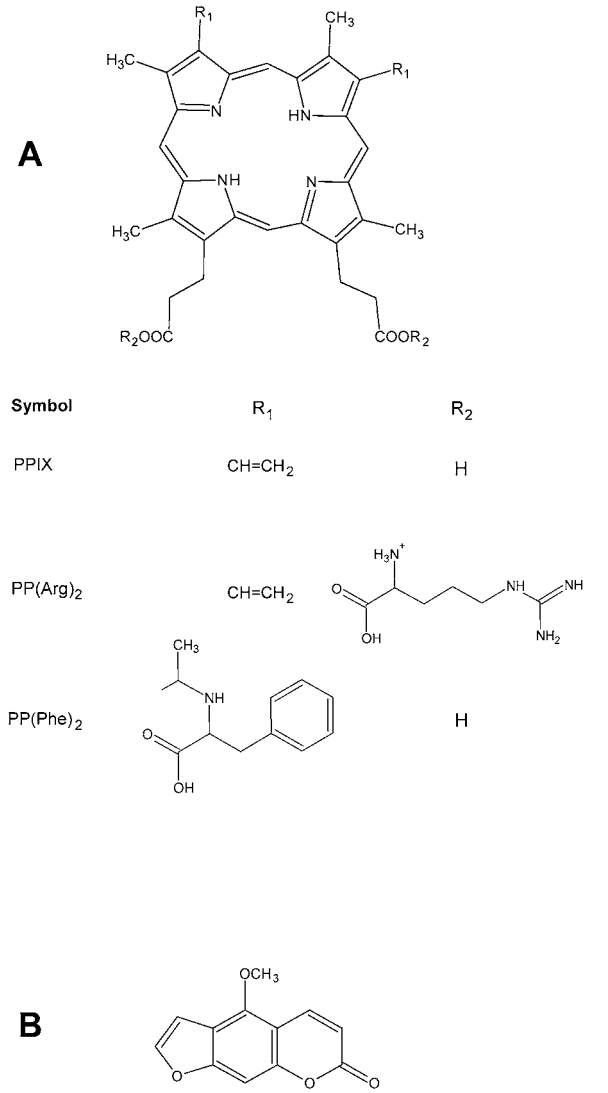

Figure 1. Chemical structure of new generation protoporphyrin sensitizers (A) and of 5-methoxypsoralen (5-MOP) (B).

demonstrated that the combination of Photofrin with 8-methoxypsoralen, a furocoumarin used in the psoralens + ultraviolet-A (UV-A) (PUVA) photochemotherapy (8), potentiates the phototoxicity of Photofrin toward murine tumor cells (9).

During the past decade, a new group of photosensitizers has been developed, which are amino acid derivatives of porphyrins (10) (Fig. 1). Along these lines, we have developed a series of protoporphyrin IX (PPIX) derivatives linking an arginyl amino group to the propionic acid side chain of the porphyrin ring. Such a combination should enhance the water solubility of the resulting photosensitizer, whereas the insertion of an amino acid chain into the vinyl bridge of PPIX compounds could increase its affinity to the tumor cells (11,12).

In this work, we have studied the photodynamic effects of two new-generation porphyrin photosensitizers toward human fibroblasts and keratinocytes in comparison with that of the parent compound, PPIX. The UV-A radiation was used here as exciting light because, in relation to other photochemotherapeutic applications in dermatology, synergistic effects of these porphyrins and bergapten (5-methoxypsoralen; 5-MOP), a furocoumarin used in the PUVA therapy of skin proliferative diseases, were also investigated.

MATERIALS AND METHODS

Cell cultures. The HS 68 normal human fibroblasts were grown in Plastic dishes, in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and containing antibiotics (100 IU penicillin and 0.1 mg mL^{-1} streptomycin) at 37°C in a 5% CO_2–humidified atmosphere. The NCTC 2544 human keratinocytes are immortalized cells derived from normal human keratinocytes. They were similarly grown using Eagle's minimum essential medium with Earle's salts (EMEM) and supplemented with the same components. All media were purchased from GIBCO (Cergy-Pontoise, France).

Preparation of photosensitizer solutions. Diarginine diprotoporphyrinate ($PP(Arg)_2$) and *N,N*-diphenylalanyl protoporphyrin ($PP(Phe)_2$) were synthesized in the Department of Biochemistry and Spectroscopy of the Military University of Technology in Warsaw, Poland (13). Stock solutions of $PP(Arg)_2$ (1.0 m*M*) were prepared in water, whereas $PP(Phe)_2$ stock solutions (1.0 m*M*) were prepared in 0.01 *M* NaOH (Merck, Darmstadt, Germany). Stock solutions of PPIX (Sigma Chemical Co., St. Louis, MO) were extempore prepared by dissolving crystals in 0.01 *M* NaOH followed by filtration of the mixture through 0.45 μm Millipore filters. The PPIX concentration was spectrophotometrically determined after dilution (7). Typically, stock solutions of about 0.4 m*M* were obtained. The UV absorption spectra of 1.0 μ*M* $PP(Arg)_2$ solution in water and of 1.0 μ*M* $PP(Phe)_2$ solutions in octanol (Sigma) and in 1% aqueous solution of Triton X-100 (Merck) to avoid aggregation of this pigment were also registered, using a UV–Vis spectrophotometer Specord M40 (Carl Zeiss, Jena, Germany). As to 5-MOP (Sigma), 1.0 m*M* stock solutions were prepared in ethanol (Merck).

Irradiation of cell cultures. For cell incubation, stock solutions of the photosensitizers were diluted in the FCS-supplemented culture medium. After 24 h of incubation, the medium was removed and the cells were rinsed twice with 1.0 mL of Hank's buffered saline solution (HBSS). Then, 1.0 mL of HBSS per petri dish was added, and the cells were irradiated either at 365 nm using a Vilber-Lourmat TFP 35L (Marne la Vallée, France) UV-A irradiation table, close to the absorption maximum of the Soret band of the porphyrin ring, or with red light ($645 < \lambda < 730$ nm). The irradiation devices have been described in detail by Tirache and Morliere (14) and Morliere *et al.* (15). Chemical actinometries based on the ferrioxalate reduction (16) or the measure of the photodegradation rate of hydroxyvinyldeuteroporphyrin chlorin in the presence of metronidazole (17) yielded dose rates equal to about 5.8 and 17.5 mW cm^{-2}, *i.e.* 1.75×10^{-8} mol s^{-1} cm^{-1} and 1.0×10^{-7} mol s^{-1} cm^{-1}, for the UV-A and red light sources, respectively.

Membrane integrity assay. After irradiation, the HBSS was removed and the irradiated cells were further incubated with DMEM or EMEM supplemented with FCS for 24 h. Then, membrane integrity was assessed using the neutral red (NR) uptake assay as described by Borenfreud and Puerner (18). In brief, the medium was removed, cells were washed twice with 1.0 mL of phosphate-buffered saline solution (PBS) and incubated for 3 h with 0.5 mL of a 0.005% NR solution in FCS-supplemented culture medium. After incubation, NR (Fluka, Saint-Quentin Fallavier, France) was removed and cells were washed three times with 1.0 mL of PBS and fixed with 0.5 mL of an aqueous solution containing 0.4% formaldehyde and 10% $CaCl_2$. Then, cells were washed again twice with PBS, and the dye was extracted with 500 μL of an aqueous solution containing 20% acetic acid and 50% ethanol. The absorbance of the resulting solutions was read at 535 nm using a 96-well microplate reader Labsystems iEMS Reader MF (Life Science International, Cergy Pontoise, France) and normalized to those of unirradiated control cells. It should be noted that the 3T3 fibroblasts photokilling assay measured by the NR uptake has been validated by the European Union for the testing of phototoxic chemicals and for the classification and labeling of hazardous chemicals (EU Commission Directive 2000/33/EC [19]).

Measurement of intracellular photosensitizer concentrations. The intracellular concentration of the photosensitizer was assessed by fluorometry according to the method described by Candide *et al.* (20). In brief, cells were disrupted with a 1% Triton X-100 (Sigma) aqueous solution. The fluorescence intensity of PPIX and $PP(Arg)_2$ was measured at $\lambda_{em} = 632$ nm ($\lambda_{ex} = 408$ nm), whereas that of $PP(Phe)_2$ was measured at $\lambda_{em} = 625$ nm ($\lambda_{ex} = 400$ nm). Concentrations were calculated from the fluorescence intensities of standard solutions in Triton X-100 (Merck). All fluorescence measurements were carried out with a Perkin-Elmer LS5 spectrofluorometer (Perkin–Elmer France, Courtaboeuf, France).

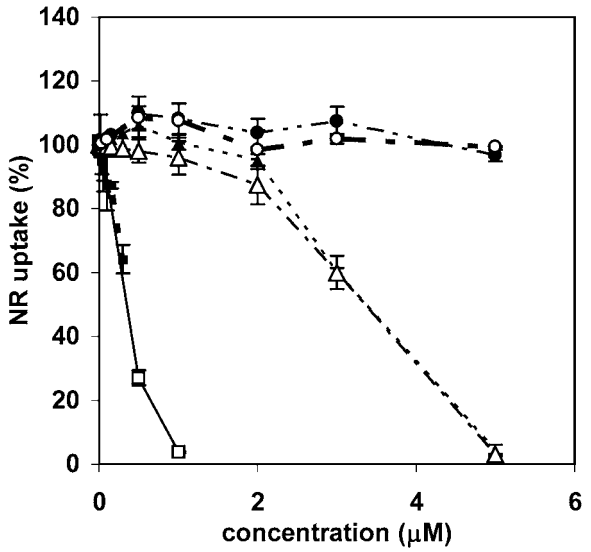

Figure 2. NR uptake by human HS 68 fibroblasts and NCTC 2544 keratinocytes treated *in vitro* with different concentrations of PPIX, $PP(Arg)_2$ and $PP(Phe)_2$ and exposed to UV-A radiation (λ = 365 nm; dose 2.0 J/cm^2, dose rate 5.8 mW cm^{-2}). The results were expressed as the percentage of absorbances at 550 nm of irradiated samples compared with that of untreated control cells. The values represent the mean ± SD of two to four experiments performed in triplicate (■ PPIX, HS 68; ▲ $PP(Arg)_2$, HS 68; ● $PP(Phe)_2$, HS 68; □ PPIX, NCTC 2544; △ $PP(Arg)_2$, NCTC 2544; ○ $PP(Phe)_2$, NCTC 2544).

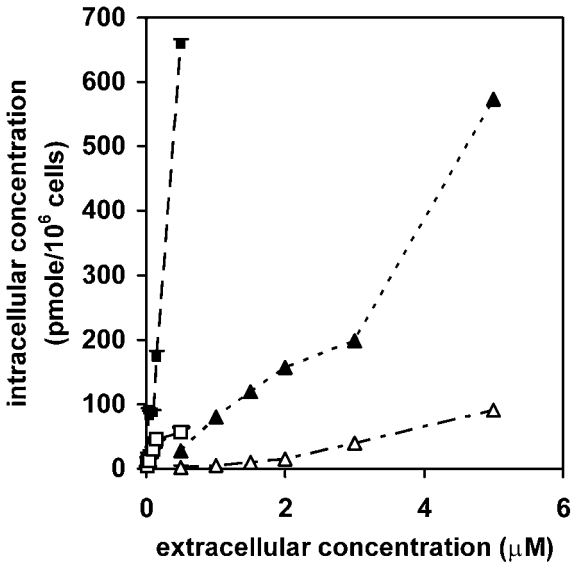

Figure 3. Cellular uptake of PPIX and $PP(Arg)_2$ into human HS 68 fibroblasts and NCTC 2544 keratinocytes treated with different concentrations of sensitizers. The intracellular concentration was measured by fluorometry. Samples were excited at λ_{ex} = 408 nm and emission was read at λ_{em} = 632 nm. Results were calculated (in pmol/10^6 cells) taking as reference the fluorescence intensity of 0.02 μ*M* of each sensitizer in 1% Triton X-100 aqueous solutions. The values represent the mean ± SD of two to three experiments performed in triplicate (■ PPIX, HS 68; ▲ $PP(Arg)_2$, HS 68; □ PPIX, NCTC 2544; △ $PP(Arg)_2$, NCTC 2544).

RESULTS AND DISCUSSION

The photocytotoxicity of PPIX or $PP(Arg)_2$ toward HS 68 fibroblasts was evaluated after incubation with various concentrations of photosensitizers followed by irradiation with 2.0 J/cm^2 of UV-A at 365 nm (Fig. 2). As described in the experimental section, the NR uptake was used as a measure of membrane integrity. Membrane integrity index can be expressed as the NR uptake by treated cells *versus* that of controls (untreated, unirradiated cells). No loss of NR uptake was observed with untreated, irradiated cells. In the absence of irradiation, no loss of membrane integrity was observed for both cell types, for incubation with all examined pigments at concentrations 1.0–7.0 μ*M* (data not shown). As can be seen in Fig. 2, under our irradiation conditions, the dose inhibiting 50% of membrane integrity (ID_{50}) is about 3.5 μ*M* $PP(Arg)_2$ in the incubation medium, whereas the corresponding dose for PPIX is about 10 times lower. In the case of NCTC 2544 keratinocytes, the NR uptake curves are very similar to those obtained with HS 68 fibroblasts. It might be anticipated that a 10-fold difference in the ID_{50} may be linked to a better uptake of PPIX by both cell types. Thus, the intracellular concentration was fluorometrically assayed after incubation with various concentration of PPIX and $PP(Arg)_2$. As can be seen on Fig. 3, the $PP(Arg)_2$ uptake is lower than that of PPIX. Incubation with 3.5 μ*M* of $PP(Arg)_2$ corresponding to the ID_{50} for the HS 68 fibroblasts or NCTC 2544 keratinocytes yields an intracellular concentration of $PP(Arg)_2$ of about 350 pmol/10^6 HS 68 fibroblasts and 50 pmol/10^6 NCTC 2544 keratinocytes. In the case of PPIX, the intracellular concentration reaches about 460 pmol/10^6 HS 68 fibroblasts and 36 pmol/10^6 NCTC 2544 keratinocytes after incubation with 0.35 μ*M* PPIX. Therefore, the incubation medium must contain 10 times more $PP(Arg)_2$ than PPIX to obtain comparable intracellular concentration of $PP(Arg)_2$ and PPIX in HS 68 fibroblasts and NCTC 2544 keratinocytes. The excellent water solubility of $PP(Arg)_2$ probably contributes to its lower effectiveness of uptake as compared with that of the extremely hydrophobic PPIX, which tends to accumulate in cell membranes as earlier shown by microspectrofluorometry (21). In support to our observation, it has been reported that the cellular uptake of porphyrins increases with their increasing lipophilicity (5,6). Furthermore, Boegheim *et al.* (22) suggested that hydrophobic groups in molecules of porphyrin sensitizers are responsible for photodynamic destruction of treated cells. It can be deduced from the combination of data in Figs. 2 and 3 that comparable ID_{50} for PPIX and $PP(Arg)_2$ correspond to a similar intracellular content of these two photosensitizers. This is apparently consistent with photophysical properties expected to be rather similar for PPIX and $PP(Arg)_2$. They have identical absorption spectrum in the irradiation wavelength range (data not shown), and their triplet state quantum yield may not be very different. However, this may be simply fortuitous because their quite opposite hydrophobic–hydrophilic behavior implies that their cellular localization and targets of photosensitization are certainly different.

The much smaller intracellular content of PPIX and $PP(Arg)_2$ in the keratinocytes as compared with that of fibroblasts (see above and Fig. 3) is intriguing especially when it can be seen from Fig. 2 that the phototoxicity of these two porphyrins is similar in both cell types. Whereas the cell size may partly account for this dramatic decrease, it is probable that other factors also contribute to this weaker uptake. However, we have no definite explanation to account for these observations, which deserve further investigation.

In contrast to $PP(Arg)_2$, the hydrophobicity of $PP(Phe)_2$ derivative seems to be comparable to that of PPIX. However, it does not accumulate in cells. Indeed, incubating HS 68 fibroblasts with 2.0 and 5.0 μ*M* of $PP(Phe)_2$ yields intracellular contents equal

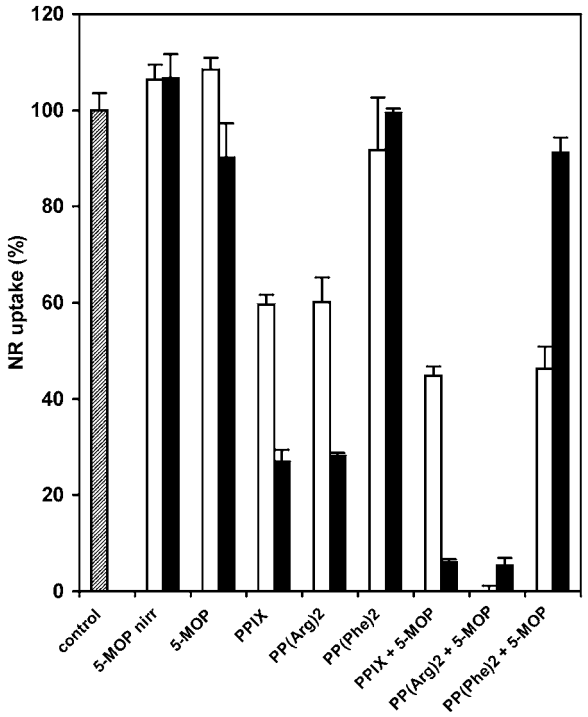

Figure 4. NR uptake by human HS 68 fibroblasts treated with 0.3 μ*M* PPIX, 4.0 μ*M* $PP(Arg)_2$ and 5.0 μ*M* $PP(Phe)_2$ and by human NCTC 2544 keratinocytes treated with 0.5 μ*M* PPIX, 3.0 μ*M* $PP(Arg)_2$ and 5.0 μ*M* $PP(Phe)_2$ alone and in the presence of 0.1 μ*M* 5-MOP and exposed to UV-A radiation (λ = 365 nm; dose 2.0 J/cm^2; dose rate 5.8 mW cm^{-2}; nirr: nonirradiated cells). Stock solutions of 5-MOP (100 μ*M*) were prepared in ethanol. The results were expressed as the percentage of absorbances at 550 nm of irradiated samples compared with that of untreated control cells. The values represent the mean ± SD of two to four experiments performed in triplicate (■ HS 68; □ NCTC 2544; nirr, nonirradiated sample).

to 12 and 17 pmol/10^6 cells, respectively, whereas no phototoxicity is observed. Similarly, intracellular concentration of $PP(Phe)_2$ in keratinocytes was 4.6 and 6.0 pmol/10^6 cells, after incubation with 2.0 and 5.0 μ*M* of $PP(Phe)_2$, respectively. The bulky phenylalanyl groups linked to the pyrrole ring of $PP(Phe)_2$ might, via steric hindrance, dramatically inhibit the uptake or the binding of sensitizer molecule to the cell membrane (or both), and, consequently, its photosensitizing activity (7,8).

Hence, the results revealed no simple correlation between phototoxic effect of the studied porphyrins and their hydrophilicity, suggesting that the UV absorption spectrum of 1.0 μ*M* $PP(Phe)_2$ in water with 1% Triton X-100 has a maximum at 386 nm (ε = 31 800 M^{-1} cm^{-1}), whereas in octanolic solution the $PP(Phe)_2$ absorption maximum lies at 397 nm (ε = 68 014 M^{-1} cm^{-1}). $PP(Arg)_2$ is practically insoluble in octanol but readily soluble in water; its absorption maximum in aqueous solution is at 473 nm (ε = 52 130 M^{-1} cm^{-1}), whereas $PP(Phe)_2$ is more soluble in octanol than in water. It is therefore difficult to link the hydrophobicity of the studied compounds to their phototoxicity toward fibroblasts and keratinocytes. In addition, the components of culture medium in which the cells were grown, such as FCS (23), may also change the solubility of the studied compounds. Apart from this, other physicochemical properties of examined pigments, such as distribution of electrical charge in molecules or steric effects of substituents should also be taken into account to explain the mechanism of the phototoxic action.

As mentioned in the Introduction, the second goal of this work was to investigate the eventual potentiation of the photocytotoxic effect of PPIX and $PP(Arg)_2$ by 5-MOP. For this purpose, after addition of the porphyrin derivative at the desired concentration, cell cultures were supplemented with 0.1, 0.25, 0.75, 1.0 or 1.5 μ*M* 5-MOP by dilution of a 100 μ*M* stock solution in ethanol and incubated as in the absence of psoralen. These concentrations were similar to 5-MOP concentrations fluorometrically detectable in human serum (0.23–2.3 μ*M* [24]) in order to be consistent with concentrations used in clinical practice. Because the stock solution was prepared in ethanol, we first check that cells incubated for 24 h in the presence of up to 1.5% ethanol alone were still fully viable before or after irradiation (data not shown). As shown in Fig. 4, 0.1 μ*M* 5-MOP alone has practically no effect on the HS 68 fibroblast or NCTC 2544 keratinocyte membrane integrity on exposure to 2.0 J/cm^2. A significant increase in the phototoxicity of PPIX or $PP(Arg)_2$ on fibroblasts was observed. Thus, in the presence of 0.1 μ*M* 5-MOP the membrane integrity of fibroblasts was decreased to about 45% on photosensitization with 0.3 μ*M* PPIX as compared with about 62% in the absence of 5-MOP. Remarkably, all cells were damaged when photosensitization was carried out with 4.0 μ*M* $PP(Arg)_2$ and 0.1 μ*M* 5-MOP (Fig. 4). The potentiation was independent of the 5-MOP concentration in the range 0.1–1.0 μ*M* (data not shown). Similarly, 0.1 μ*M* 5-MOP potentiated the phototoxic effect of 0.5 μ*M* PPIX or 3.0 μ*M* $PP(Arg)_2$ toward keratinocytes, leading to a decrease in the membrane integrity index from ~27% to ~6% and from ~28% to ~5%, respectively (Fig. 4). Interestingly, on addition of 0.25 and 0.5 μ*M* of 5-MOP, a potentiation of the phototoxicity of 3.0 μ*M* $PP(Arg)_2$ toward keratinocytes was observed (NR uptake was about 16 and 13%, respectively). On the other hand, the potentiation was inhibited at higher 5-MOP concentration, so that NR uptake increased from ~54 to ~93% (Fig. 5). Incidentally, it should be noted that 5.0 μ*M* $PP(Phe)_2$, which alone is not phototoxic to fibroblasts, becomes phototoxic in the presence of 0.1 μ*M* 5-MOP, decreasing the NR uptake (membrane integrity index) of fibroblasts from about 102 to about 36% (Fig. 4). Little, if any, potentiation of the phototoxicity of $PP(Phe)_2$ was observed with keratinocytes.

It is difficult to explain the mechanism by which 5-MOP influences the phototoxic effect of the studied porphyrins. Fluorometric study reveals that the intracellular $PP(Arg)_2$ concentration decreases on 5-MOP addition. The decrease in the $PP(Arg)_2$ intracellular content is accompanied by an inhibition of the phototoxic effect toward NCTC 2544 keratinocytes (Fig. 5). However, this inhibition is observed only for high 5-MOP concentration (from 0.75 to 1.5 μ*M*), whereas after incubation with 0.25–0.5 μ*M* 5-MOP the phototoxicity of $PP(Arg)_2$ is potentiated despite a decrease in its intracellular concentration. On the other hand, the intracellular $PP(Arg)_2$ concentration in NCTC 2544 keratinocytes and that of PPIX and $PP(Phe)_2$ in HS 68 fibroblasts and NCTC 2544 keratinocytes cells do not significantly change in the presence of 0.1 and 0.25 μ*M* 5-MOP (Table 1). In this context, the potentiation of the porphyrin photodynamic effect by low 5-MOP concentrations (≤0.5 μ*M*) might be explained by an increase in the photosensitizing efficiency of porphyrins or by the induction of an intrinsic phototoxic effect of 5-MOP under the influence of porphyrin sensitizers. As reported by Sousa *et al.* (9), at such low UV-A doses, psoralens alone do not kill the cells. However, under these conditions they could produce cytotoxic photoperoxidation products, whose concentration markedly increases in the presence of porphyrins (9). Although these previous

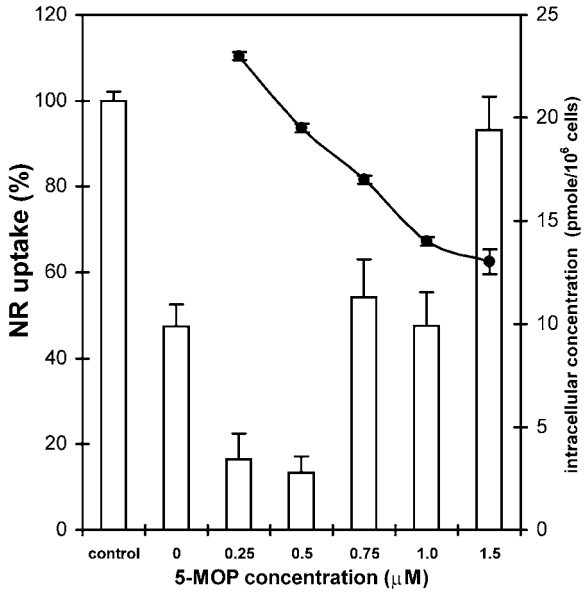

Figure 5. NR uptake by human NCTC 2544 keratinocytes treated with 3.0 μM $PP(Arg)_2$ in the absence or in the presence of 5-MOP for different concentrations (0.25–1.5 μM) and exposed to UV-A radiation ($\lambda = 365$ nm; dose 2.0 J/cm^2; dose rate 5.8 mW cm^{-2}). The full line corresponds to cellular uptake of $PP(Arg)_2$. The results were expressed as the percentage of absorbances at 550 nm of irradiated samples compared with that of untreated control cells. The concentration of photosensitizer in cells was measured by fluorometry as described in Fig. 3. The values represent the mean ± SD of two to three experiments performed in triplicate.

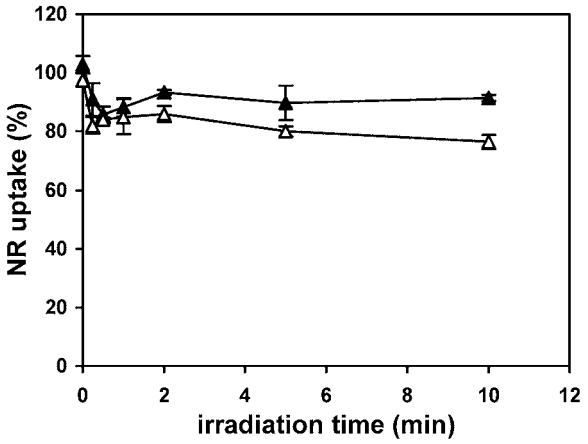

Figure 6. NR uptake by cultured human HS 68 fibroblasts treated with 2.0 μM $PP(Arg)_2$ alone and in the presence of 0.1 μM 5-MOP after exposure to various doses of red light ($645 < \lambda < 730$ nm; dose rate 17.5 mW cm^{-2}). The results were expressed as the percentage of absorbances at 550 nm of irradiated samples compared with that of untreated control cells. The values represent the mean ± SD of two experiments performed in triplicate. (▲ $PP(Arg)_2$; △ $PP(Arg)_2$ + 5-MOP).

results were obtained with higher concentrations of 5-MOP (50–100 μM), there is no reason to exclude a similar mechanism of action of 5-MOP during its application in the 0.5–7.0 μM concentration range used in our experiments. To shed some light on the mechanism(s) of the potentiation, cells treated with 2.0 μM $PP(Arg)_2$ were irradiated with red light to avoid any direct photosensitization by 5-MOP. Under these conditions, the membrane integrity index of fibroblasts slightly decreased from ~92 to ~76% in absence and in presence of 5-MOP, respectively (Fig. 6), suggesting that the psoralen needs no excitation by UV-A light to induce some potentiation. It may be speculated that, under UV-A irradiation, the perturbation of cell membrane structures by the hydrophobic 5-MOP, a well-known membrane localizing molecule (25), may contribute, at least in part, to the potentiation of the porphyrin photosensitization. Specifically, the modification of cell membranes by 5-MOP may aggravate, in a synergistic manner, damage due to porphyrin-photosensitized membrane lipid peroxidation (8,26). On the other hand, it does not exclude that the high hydrophobicity of 5-MOP may favor the formation of photoadducts after complexation to nucleic acids (27). The production of reactive oxygen species should also be examined in further research. Until now, there is no evidence that the synergistic effect of 5-MOP involves another mechanism than that of 8-MOP in combination with Photofrin. However, the results suggest that the mechanism of interactions between 5-MOP and $PP(Arg)_2$ may change with increasing 5-MOP concentrations particularly in the case of keratinocytes (Fig. 5).

In conclusion, this *in vitro* study has indicated that $PP(Phe)_2$, a new-generation, water-insoluble porphyrin photosensitizer, exhibits no significant phototoxic effect toward healthy human fibroblasts and keratinocytes both alone and in combination with 5-MOP. In contrast, another porphyrin derivative, $PP(Arg)_2$, much more hydrophilic and easier to handle than PPIX, showed a weaker potential phototoxicity on the studied skin cells than PPIX. However, in combination with 5-MOP, the photodynamic effect of $PP(Arg)_2$ was similar to that of PPIX. It may be interesting to study in animal models whether synergistic phototoxic effects are observed when $PP(Arg)_2$ and 5-MOP are applied together during skin cancer treatment. An evident argument against the use of 5-MOP is its well-established photocarcinogenicity (28). Two comments should be made in this regard. First, the potentiation of the photodynamic effects observed here occurred at very low 5-MOP concentrations,

Table 1. Intracellular concentration (as pmol/10^6 cells) of PPIX, $PP(Arg)_2$ and $PP(Phe)_2$ in HS 68 fibroblasts and NCTC 2544 keratinocytes in the absence or presence of 5-MOP. Each value represents the mean ± SD of two to three experiments performed in triplicate

HS 68					
0.3 μM PPIX	0.3 μM PPIX + 0.1 μM 5-MOP	2.0 μM $PP(Arg)_2$	2.0 μM $PP(Arg)_2$ + 0.1 μM 5-MOP	5.0 μM $PP(Phe)_2$	5.0 μM $PP(Phe)_2$ + 0.1 μM 5-MOP
394.0 ± 23.0	383.0 ± 16.0	157.0 ± 4.0	112.9 ± 3.3	17.0 ± 5.0	12.0 ± 3.0
NCTC 2544					
0.5 μM PPIX	0.5 μM PPIX + 0.1 μM 5-MOP	3.0 μM $PP(Arg)_2$	3.0 μM $PP(Arg)_2$ + 0.1 μM 5-MOP	5.0 μM $PP(Phe)_2$	5.0 μM $PP(Phe)_2$ + 0.1 μM 5- MOP
56.0 ± 5.0	48.0 ± 2.0	40.0 ± 3.0	35.7 ± 1.4	6.0 ± 0.6	5.7 ± 1.0

which may considerably decrease the risk of photocarcinogenicity. Second, if the photocarcinogenicity of 5-MOP is still demonstrated in the porphyrin–5-MOP combination photochemotherapy, an evaluation of the risk/benefit ratio should be assessed before rejecting 5-MOP as an adjuvant drug.

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