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João Nuno Silva, Antoine Galmiche, João P.C. Tomé, Agnès Boullier, Maria G.P.M.S. Neves, et al.. Chain-dependent photocytotoxicity of tricationic porphyrin conjugates and related mechanisms of cell death in proliferating human skin keratinocytes. *Biochemical Pharmacology*, 2010, 80 (9), pp.1373. 10.1016/j.bcp.2010.07.033 . hal-00623305

HAL Id: hal-00623305

<https://hal.science/hal-00623305>

Submitted on 14 Sep 2011

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Accepted Manuscript

Title: Chain-dependent photocytotoxicity of tricationic porphyrin conjugates and related mechanisms of cell death in proliferating human skin keratinocytes

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PII: S0006-2952(10)00571-X
DOI: doi:10.1016/j.bcp.2010.07.033
Reference: BCP 10666

To appear in: *BCP*

Received date: 14-6-2010
Revised date: 20-7-2010
Accepted date: 23-7-2010

Please cite this article as: Silva JN, Galmiche A, Tomé JPC, Boullier A, Neves MGPMS, Silva EMP, Capiod J-C, Cavaleiro JAS, Santus R, Mazière J-C, Filipe P, Morlière P, Chain-dependent photocytotoxicity of tricationic porphyrin conjugates and related mechanisms of cell death in proliferating human skin keratinocytes, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2010.07.033

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4 **Chain-dependent photocytotoxicity of tricationic porphyrin conjugates**
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7 **and related mechanisms of cell death in proliferating human skin keratinocytes**
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47
48 The abbreviations used are: 3-MA, 3-methyladenine; ALA, 5-aminolevulinic acid; BCC, Basal cell carcinoma;
49 CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EMEM, minimum
50 essential medium with Earle's salts; FCS, fetal calf serum; GFP, Green fluorescent protein; HBSS, Hanks'
51 balanced saline solution; LC3, microtubule-associated protein light-chain 3; MAL, methyl aminolevulinic
52 acid; NR, neutral red; DPBS, Dulbecco's phosphate-buffered saline; PDT, photodynamic therapy; PI,
53 propidium iodide; PPIX, protoporphyrin IX; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate;
54 TBARS, thiobarbituric acid reactive substances; TX100, triton X100.
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ABSTRACT

Photodynamic therapy (PDT) is a poor treatment option for nodular basal cell carcinomas and squamous cell carcinomas. As a result, the search for new photosensitizers with better effectiveness is of current interest. The photocytotoxicity of conjugates (P-R) of a water-soluble tricationic porphyrin (P-H) having similar efficiency of production of singlet oxygen, the PDT cytotoxin, has been assessed *in vitro*. Links between uptake, intracellular localization, photooxidative stress, photocytotoxicity and ability to induce programmed cell death are established. Conjugates bearing methyl (P-Me), Di-O-isopropylidene- α -D-galactopyranosyl (P-OGal) or *N,N'*-dicyclohexylureidooxycarbonyl (P-DDC) chains are efficiently taken-up by proliferating NCTC 2544 keratinocytes. The relative order of photocytotoxicity is P-OGal > P-DDC = P-Me >> P-H. The photocytotoxic potential of P-Me, P-OGal and P-DDC equals that of endogenous protoporphyrin IX induced by δ -aminolevulinic acid or its esters, the pro-drugs currently employed for PDT of skin lesions. Microfluorometry shows that P-Me, P-OGal, and P-DDC localize in endocytotic or pinocytotic vesicles but not in mitochondria or nucleus. Absence of annexin V binding, caspase activation or chromatin condensation suggests that cell photosensitization by P-R does not induce apoptosis. On the other hand, P-OGal photocytotoxicity correlates with appearance of multiple vesicles that have hallmarks of autophagy compartments, being decorated with the marker LC3 in cells transfected with an expression vector encoding GFP-LC3. p38 and JNK phosphorylation and inhibition of ERK1/2 phosphorylation suggest close relationship between mortality of NCTC 2544 keratinocytes and MAPK pathway impairment. Given their potentially easy formulation, water-soluble P-R are promising powerful photosensitizers for PDT of skin lesions.

Keywords: PDT, photocytotoxicity, necrosis, autophagy, caspases, MAPK pathway.

1. Introduction

The treatment of malignant or benign skin diseases by photodynamic therapy (PDT) has developed rapidly [1]. Current evidence indicates that topical PDT is effective in actinic keratosis, Bowen's disease and basal cell carcinoma (BCC) but it is a relatively poor treatment option for either squamous cell carcinomas or nodular BCC. In current skin PDT, topical application of δ -aminolevulinic acid (ALA) or its methyl ester (MAL), precursors of protoporphyrin IX (PPIX), is followed by illumination of the diseased area with red light. The poor water solubility of these agents requires expensive preparation of formulations for systemic or topical administration. Hence, development of new photosensitizers with better effectiveness is of current interest. Such new photoactive substances might demonstrate better clinical and histological efficacy in treating skin lesions for which topical MAL-PDT is ineffective (e.g. melanoma) or might be used as a palliative treatment for patients with plaque-type cutaneous T-cell lymphoma. Despite superior absorbance in the 700 nm region (molar absorptivity: $\sim 2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) phthalocyanines are not currently used in the PDT of epidermal skin lesions (normally $\sim 1\text{-}2$ mm thickness). They tend to form aggregates of reduced uptake and photosensitizing properties and the phthalocyanine ring resists to biodegradation [2]. However, their encapsulation in silica nanoparticles would appear to provide a promising approach to photosensitizer uptake and delivery as it has already been shown to improve the aqueous solubility and stability of the silicon phthalocyanine 4 [3]. For these reasons, the synthesis of water-soluble derivatives of porphyrins and chlorins has been investigated. Conjugation of amino-acids, peptides and sugars to photosensitizers favours specific targeting since such groups play key roles in the recognition and metabolism in micro-organisms and cells [4, 5].

In this study, new water-soluble derivatives of 5-(4-carboxyphenyl)-10,15,20-tris(4-

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4 methylpyridinium-4-yl)porphyrin tri-iodide (P-R), whose absorbance maxima are shifted to the
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6 red by 15 to 20 nm as compared to PPIX, have been shown to be of potential interest in derma-
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8 tological PDT since a poly-S-lysine conjugate is, *in vitro*, an effective photosensitizer towards
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10 rapidly proliferating human skin keratinocytes [6]. Furthermore, tricationic porphyrins are prom-
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12 ising molecules since they may bind to negatively charged skin cells after disruption of the *Strat-*
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14 *tum Corneum* barrier. In this study, the photocytotoxicity effectiveness of conjugates with a
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16 methyl (P-Me), a dicyclohexylureidooxy group (P-DDC), a di-*O*-isopropylidene- α -D-
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18 galactopyranosyl group (P-OGal), a α/β -D-galactopyranosyl group (P-Gal) has been evaluated in
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20 cultured proliferating human skin keratinocytes for allowing the direct comparison of results ob-
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22 tained with the poly-S-lysine conjugate (P-(Lys)_n) [6]. We have also determined the molecular
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24 mechanisms involved in the photo-induced cell death, the induction of a specific death mode be-
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26 ing susceptible to result in a therapeutic advantage.
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33 **2. Materials and Methods**

34 **2.1. Chemicals, culture media and routine spectroscopic equipment**

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37 Reagents for cell culture, minimum essential medium with Earle's salts (EMEM), Hanks' ba-
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39 lanced saline solution containing 20 mM Hepes (HBSS), Dulbecco's phosphate-buffered saline
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41 (DPBS), all without phenol red, fetal calf serum (FCS), trypsin and antibiotics were purchased
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43 from Sigma Chemical Co (St Louis, MO, USA). Chemicals and reagents were obtained from
44
45 Sigma chemical Co, Merck (Darmstadt, Germany), Fluka (Saint-Quentin Fallavier, France) and
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47 Molecular Probes (Eugene, OR, USA) [6]. Inhibitors of p38 (SB203580) and JNK (SP600125)
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49 were purchased from Calbiochem (Darmstadt, Germany). Specific antibodies against caspase-3,
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51 caspase-8, caspase-9 and p62/SQSTM1 were provided by Santa Cruz Biotechnology (Heidelberg,
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53 Germany) whereas those against total p38, phospho-p38, total JNK, phospho-JNK, total Akt,
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4 phospho-Akt, total ERK, phospho-ERK were purchased from Cell Signaling (Beverly, MA,
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6 USA). The synthesis of the porphyrin conjugates (P-R) (Fig. 1) was performed as described by
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8 Tomé et al. [5, 7]. The 500 μ M stock solutions were prepared in water:DMSO (1:1 v/v) and
9
10 stored at 0-4 °C. A UVIKON 943 spectrophotometer from Kontron Instruments (Montigny les
11
12 Bretonneux, France) was used for optical absorption spectrophotometry. Fluorescence measure-
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14 ments were carried out with a SLM AMINCO-BOWMAN (series 2) fluorometer (Bioritech,
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16 Chamarande, France).
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21 **2.2. Cell culture and treatment**

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23 The immortalized NCTC 2544 human skin keratinocyte cell line was purchased from ICN Flow
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25 (Fontenay sous Bois, France). Cells were propagated, grown and seeded as earlier described [6].
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27 For fluorescence microscopy, cells were seeded on 30 mm diameter and 0.15 mm thick cover-
28
29 glass at a density of about 1,700 cells/cm² (2.5 mL at about 5,000 cells/mL) and were grown for 4
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31 days. The incubations were carried out with 1 mL of P-R at various concentrations and for vari-
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33 ous times in either HBSS or 10% FCS-supplemented EMEM. In the case of endogenous PPIX
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35 synthesis, incubations were performed with 0.2 to 0.8 mM ALA in 10% FCS-supplemented
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37 EMEM. After incubation, cells were washed twice with 4 mL of HBSS before measurements.
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43 **2.3. Irradiation**

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45 Washed cell monolayers, covered with 1mL of HBSS without photosensitizer, were irradiated
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47 with broad band red light provided by a custom-built table consisting of two 300 watt tungsten-
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49 halogen lamps whose light was filtered with Balzers Y54 and calflex 3000 optical filters [6, 8].
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51 Petri dishes were placed on a 30 x 25 cm thermostated (37 °C) glass table above the lamps. Un-
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53 der these conditions, most of the light originates from wavelengths in the range 500 - 750 nm as
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55 determined with a CS 1000A Minolta spectroradiometer. The lamp actinometry is described in
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57 the supplementary data.
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2.4. Determination of intracellular photosensitizer concentration

Immediately after P-R or ALA incubation and washings, cells were mechanically scrapped in 1 mL of water. After collection, 100 μ L of an 11% SDS solution in 10 mM phosphate buffer (pH 7.0) were added to the disrupted cell suspension. Fifty μ L of this solution were saved for protein determination using Lowry's method with Folin reagent. The remainder was utilized for fluorometric measurement of photosensitizer concentration, using standard P-R solutions for calibration ($\lambda_{exc} = 427$ nm and $\lambda_{em} = 659$ nm) or standard PPIX solutions ($\lambda_{exc} = 407$ nm and $\lambda_{em} = 636$ nm) [9]. Data are the mean \pm SD of at least three independent experiments, each performed in triplicate.

2.5. Neutral Red uptake assay

The assessment of photocytotoxicity by the NR uptake assay has been validated by the European Union for the testing of phototoxic chemicals and for the classification and labelling of hazardous chemical (EU Commission Directive 2000/33/EC). This assay has been fully described by Filipe et al. [10].

2.6. Thiobarbituric acid reactive substances (TBARS) assay

Immediately after irradiation or sham-irradiation, 0.9 mL of supernatants was collected. After addition of 90 μ L of butylated hydroxytoluene (2% w/w in ethanol) samples were kept frozen until TBARS assay. Cells were washed twice with DPBS, scrapped and collected for protein determination. TBARS were fluorometrically determined in terms of malondialdehyde equivalent as earlier described [6].

2.7. Fluorescence microscopy equipment for photosensitizer localization

An inverted fluorescence microscope (Eclipse TE 2000 DV Nikon) equipped with a CoolS-NAP_{HQ}TM detector cooled to -30 °C (Princeton Instruments; Division of Roper Scientific, Evry, France) was used for cell microfluorometry. This system is controlled by the Meta-

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4 view/Metamorph software, which is also used for image analyses. The fluorescence recording
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6 conditions (objective, camera binning and exposure time, dichroic mirrors, filters) are fully de-
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8 scribed in ref [6]. No porphyrin fluorescence was detected with the green emission filter while
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10 negligible fluorescence of the LysoTracker Green was recorded with the red emission filter.
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12 Background fluorescence obtained from cell-free areas was subtracted from all the acquired im-
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14 ages.
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18 **2.8. Flow cytometry**

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20 At various times after photosensitization, adherent cells were washed twice in ice-cold DPBS and
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22 detached with Accutase™ (Sigma-Aldrich Co). Cells were transferred to 15 mL conical tubes and
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24 gently washed with serum supplemented medium by low speed centrifugation at 1500 rpm for 5
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26 minutes. Cell pellets were washed once with DPBS and then re-suspended in assay buffer (0.1M
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28 HEPES/NaOH, pH7.4; 140mM NaCl; 25mM CaCl₂) at a concentration of 1×10^6 cells/mL. An-
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30 nexin V-FITC binding was assessed using the Santa Cruz Biotechnology Apoptosis Kit (Santa
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32 Cruz Biotechnology, Heidelberg, Germany) according to the manufacturer's protocol. After 15
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34 min of incubation in the dark with annexin V-FITC (5 µg/mL) and propidium iodide (PI) (5
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36 µg/mL), cell fluorescence was measured in the spectral regions 505-545nm (FL1) for A and 610-
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38 650nm (FL3) for PI with a Cytomics FC 500 flow cytometer (Beckam Coulter, Villepinte,
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40 France). Data analysis was performed using Cytomics Analysis software.
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48 **2.9. DNA fragmentation assay**

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50 DNA fragmentation was assessed through determining cytoplasmic accumulation of histone-
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52 associated DNA fragments by an enzyme-linked immunosorbent assay (Roche Diagnostics,
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54 Mannheim, Germany). At different times after irradiation, the cell supernatant was removed, cells
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56 were washed twice with ice-cold DPBS and lysed with the kit buffer. Equal amounts of protein
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58 from the cytoplasmic fraction (100 µg, Bradford assay from Bio-Rad Laboratories, Marne-la-
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4 Coquette, France) were transferred into an anti-DNA precoated microtiter plate and analyzed
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6 using the ELISA procedure recommended by the manufacturer.
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9 **2.10. Caspase assays**

10 After irradiation cells were lysed in buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS,
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12 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, supplemented with 10 µg/mL
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14 pepstatin, aprotinin and leupeptin), sonicated and centrifuged at 14 000×g for 5 min at 4 °C. Su-
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16 pernatants were saved as representative cytosol extracts. Aminomethylcoumarin (AMC)-
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18 conjugated peptide substrates at a final concentration of 20 µM were added to 40 µg of total cyto-
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20 sol protein and incubated for 30 min at 37 °C. The specific substrates used were: acetyl-Asp-Glu-
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22 Val-Asp-AMC (Ac-DEVD-AMC) for caspase-3, acetyl-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC)
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24 for caspase-8 and acetyl-Leu-Glu-His-Asp-AMC (Ac-LEHD-AMC) for caspase-9 (AnaSpec, Inc,
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26 San Jose, CA, USA). The fluorescence of coumarin dyes was measured with a 96-microwell plate
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28 reader under excitation at 360 nm and emission at 460 nm.
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36 **2.11. Immunoblot analysis**

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38 Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. The
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40 protein concentration of cell extracts was determined using the Pierce BCA assay (Thermo Fisher
41
42 Scientific, Cergy Pontoise, France) and equal amounts of proteins (40 µg) were separated by 12%
43
44 SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were immunoblotted
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46 using conventional procedures and revealed using an enhanced chemiluminescence detection kit
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48 from Amersham (Saclay, France). Western blots were scanned and quantified using the software
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50 ImageJ (National Institutes of Health).
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54 **2.12. Cell transfection with GFP-LC3**

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56 The construct encoding microtubule-associated-protein-light-chain-3 (LC3) protein tagged at its
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58 N-terminus with green fluorescent protein (GFP), GFP-LC3, has been used to monitor autophagy
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4 through direct fluorescence microscopy and was provided by Drs. T. Yoshimori and N. Mizushima
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6 (Department of Bioregulation and Metabolism, The Tokyo Metropolitan Institute of Medical
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8 Science, Japan) [11]. NCTC 2544 cells grown on glass coverslips were transfected with GFP-
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10 LC3 using Lipofectamine 2000 according to the supplier's protocol (Invitrogen). After transfec-
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12 tion, cells were incubated for 3 hours with 5 μ M P-OGal or for 18 h with 0.8 mM ALA and irra-
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14 diated for 15 min. At various times after irradiation, cells were fixed with paraformaldehyde,
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16 rinsed and mounted on glass slides using Mowiol (Calbiochem). Cells were observed under a
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18 Nikon Eclipse TE2000-U fluorescence microscope equipped with a plan APO VC 60X / 1.40 ob-
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20 jective under oil immersion. Cells with active autophagy were defined as those displaying 3 or
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22 more puncta of GFP-LC3. Results are given under each experimental condition as percentage of
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24 cells with more than 3 puncta of GFP-LC3, and are based on the counting of over 500 cells ex-
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26 pressing GFP-LC3.
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32 33 **2.13. Cytochrome c immunofluorescent staining**

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35 Cells grown on glass coverslips and treated as indicated were washed with PBS, fixed with 4%
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37 paraformaldehyde , and permeabilized with 0.1% TX100. Immunolabelling was performed using
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39 a mouse antibody raised against cytochrome c (BD Bioscience, Le Pont de Claix, France) and a
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41 secondary antibody labeled with Cy3 (Jackson Immunoresearch). Nuclei were counterstained
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43 with DAPI. The stained samples were rinsed and mounted in Mowiol (Calbiochem) and the slides
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45 were examined and photographed using a Nikon Eclipse TE2000-U fluorescence microscope
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47 equipped with a plan APO VC 60X / 1.40 objective under oil immersion.
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52 53 **2.14. Statistical analyses**

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55 Statistical analyses were performed with the Student's t-test. Statistical significances are provided
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57 in the figures (* $p < 0.05$, ** $p < 0.01$).
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3. Results

3.1. Uptake of P-R derivatives by cells

NCTC 2544 keratinocytes have been incubated at various durations with increasing concentrations of porphyrins in FCS-supplemented EMEM. As shown in Fig. 2A, the uptake of all derivatives is extremely fast and reaches a plateau within less than an hour. No further uptake is observed with incubation of up to 18 h. P-H and, to a lesser extent, P-Gal are poorly incorporated when compared to P-Me, P-DDC and P-OGal. After a 3 h incubation in FCS-supplemented EMEM, the uptake increases almost linearly as a function of the incubation concentration for all derivatives (Fig. 2B). The relative order of magnitude of the uptake is the same as that observed in Fig. 2A.

Though incubation in FCS-supplemented culture medium is more relevant to the *in vivo* situation, incubation in serum-free HBSS has also been performed to determine whether serum proteins play any peculiar role in the uptake process. The P-R uptake profiles as a function of incubation time or of incubation concentration are found to be similar in the presence and absence of serum (data not shown). This suggests that, in contrast to the behavior of P-(Lys)_n, there is no strong interaction between the other P-R shown in Fig. 1 and serum proteins [6].

3.2. Photocytotoxicity of P-R derivatives

To reach maximum uptake, cells were incubated for 3 h with porphyrins in FCS-supplemented EMEM prior to irradiation. No cytotoxicity was observed with untreated sham-irradiated cells or with untreated irradiated (up to 60 min) cells in the presence of 0.5% or less DMSO in the incubation medium (data not shown). Moreover, none of these porphyrins exhibited dark cytotoxicity at incubation concentrations up to 5 μ M. Figure 2C (irradiation with increasing light doses) demonstrates that P-Me, P-DDC and P-OGal all exhibit a much larger photocytotoxicity than P-H

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4 or P-Gal at an incubation concentration of 5 μ M. Conversely, lower concentrations of P-Me, P-
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6 DDC and P-OGal are sufficient to achieve high photocytotoxicity (Fig. 2D). The incubation con-
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8 centrations necessary to obtain a 50% decrease in NR uptake after a 30 min irradiation can be
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10 estimated from Fig. 2D. These values, hereafter referred to as "photocytotoxic concentrations"
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12 (PC_{50}), are reported in Table 1, which also includes earlier data obtained with the P-(Lys)_n deriva-
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14 tive. Table 1 provides another photocytotoxicity index, the "intracellular phototoxic concentra-
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16 tion" (IPC_{50}) which represents the intracellular concentration which produces a 50% decrease in
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18 NR uptake after a 30 min irradiation. Low IPC_{50} values of ~50 pmol per mg of protein are ob-
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20 tained with P-Me, P-DDC or P-OGal and a relative efficiency -- P-OGal > P-DDC = P-Me >> P-
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22 Gal > P-H -- is deduced from Table I. Experiments performed after incubation in the absence of
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24 serum proteins lead to similar data for these conjugates, further illustrating that little if any role is
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26 played by serum proteins in uptake of these porphyrins (data not shown).
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33 **3.3. Oxidative stress photo-induced by P-R derivatives**

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35 Singlet oxygen is the main cytotoxin involved in PDT with porphyrins [12]. The photophysical
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37 studies here revealed no major difference in the efficiency of P-H, P-Me, P-DDC, P-OGal and P-
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39 Gal to produce singlet oxygen in a high yield whereas P-(Lys)_n was found earlier to be about 4
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41 times less effective [6, 13]. Inhibition of cell photosensitization by specific ¹O₂ quenchers such as
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43 N₃⁻ ions (Table 1) demonstrates the role of singlet oxygen in photo-oxidative stress induced by
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45 the tricationic porphyrins. Lipid peroxidation has been used as an overall index of oxidative
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47 stress. It has been quantified by measuring TBARS formation in cell supernatants since previous
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49 studies demonstrate practically no intracellular TBARS formation in photosensitized cells them-
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51 selves. The TBARS production is shown to increase nearly linearly with the irradiation time (Fig.
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53 3A). The ability of P-R to trigger lipid peroxidation parallels their photocytotoxic potential, and
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55 the strongly photocytotoxic P-Me, P-DDC and P-OGal as well as P-(Lys)_n (see Fig. 4 in [6]), may
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4 be seen to be the most effective TBARS producers. Again, similar data were obtained in the ab-
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be seen to be the most effective TBARS producers. Again, similar data were obtained in the absence of serum proteins (data not shown).

3.4. Cytotoxicity and oxidative stress photo-induced by PPIX

To compare the effects of P-R photosensitization to those of ALA, the pro-drug used in the PDT of skin lesions, cells have been incubated with various concentrations of ALA prior to irradiation in order to induce endogenous PPIX. Subsequently, the intracellular PPIX content was determined by fluorescence measurement. The photocytotoxicity or the lipid peroxidation, or both have also been determined. Data in Fig. 3B show that the IPC_{50} is approximately 50 pmol per mg of protein. About 1.8 mol of TBARS per mol of intracellular PPIX are released after a 30 min irradiation (Fig. 3C).

3.5. Fluorescence microscopy of P-R derivatives

P-R derivatives fluoresce near 660 nm and exhibit comparable fluorescence quantum yields under similar solvent conditions [13]. Figure 4 illustrates the phase contrast and fluorescence images obtained for control cells (sham-treated cells) and for cells treated for 3 h with P-R in FCS-supplemented culture medium. The fluorescence images have been obtained with an excitation in the near UV (330-380 nm) and an emission in the red-visible range (610-700 nm). Figures 4G to R are matching fluorescence images of the phase contrast images (Figs. 4A-F). For ready comparison, fluorescence images are displayed on the same scale in panels G to L, while they are displayed between the minimum and maximum fluorescence levels in M to R. As expected, untreated cells exhibit a very low fluorescence arising from the cytoplasm with larger intensities in the perinuclear area and the mitochondrial network. Cells treated with P-Me, P-DDC and P-OGal fluoresce more intensely, in agreement with greater uptake. A rather diffuse fluorescence with some spots in the perinuclear area is observed from cells treated with P-H and P-Gal, suggesting a main cytoplasmic and plasma membrane localization and little accumulation at specific sites.

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4 By contrast, cells treated with P-Me, P-DDC and P-OGal exhibit stronger fluorescence, mostly
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6 localized in perinuclear spots which suggests more specific localization in lysosomes or lyso-
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8 some-like structures. A double labeling of the cells (Figs. 5A,B,C) was performed with P-R and
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10 LysoTracker Green, a lysosome specific probe emitting a green fluorescence centered toward 510
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12 nm. As shown for P-DDC (Figs. 5B,C) the localization patterns of the green and red fluores-
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14 cences are very similar, although a total superimposition cannot be achieved. This suggests a lo-
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16 calization in other non-lysosomal structures, most probably, endocytotic or pinocytotic vesicles
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18 beside a minor lysosomal incorporation. These vesicles have a pH higher than that of lysosomes
19
20 (pH 5-6) and are therefore inefficiently labeled by LysoTracker Green[®]. On the other hand, lyso-
21
22 somal staining by the probe may be partially hidden by FRET from the probe (the donor) to P-R
23
24 (the acceptor). Such behavior is observed in the case of another PDT model where tolyporphin is
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26 the photosensitizer [8]. Since P-R are positively charged, and since the inner mitochondrial mem-
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28 brane exhibits a negative potential, mitochondria are expected to be a preferential site for P-R
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30 localization. However, micrographs obtained with the specific mitochondrial probes, rhodamine
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32 123 and MitoTracker[®] show a perinuclear network extending all over the NCTC 2544 keratino-
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34 cytes (see also Gaullier et al. [14]) incompatible with the pattern of P-Me, P-DDC and P-OGal
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36 localization (Figs. 5D,E,F). This observation confirms previous results obtained with P-(Lys)_n
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38 [6]. Although tetracationic porphyrins readily accumulate in the cell nucleus [15, 16], the fluores-
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40 cence depression on the nuclear area observed for all P-R strongly suggests very little, if any,
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42 localization in the nucleus of NCTC 2544 keratinocytes.
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52 **3.6. Mechanisms of the PDT-induced death in NCTC 2544 cells**

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54 Necrosis induced *in vitro* by PDT with porphyrins has long been established, and is essentially
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56 characterized by increased cell volume, organelle swelling and plasma membrane alterations
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58 [17]. Apoptosis and autophagy are induced by a variety of stress factors, notably those encoun-
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4 tered in cancer therapies. Two reviews have been recently published on guidelines concerning the
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6 use and interpretation of assays for determining mechanisms of cell death [18, 19]. It is of interest
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8 to understand the death mechanisms underlying the efficient photocytotoxicity of P-Me, P-OGal
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10 and P-DDC. As P-Me, P-OGal and P-DDC exhibit similar intracellular localization, P-OGal --
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12 the most effective photosensitizer -- was chosen as representative P-R for this study.
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15 16 **3.7. Is apoptosis involved in P-R photocytotoxicity?**

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18 Translocation of phosphatidylserine from the inner layer to the outer layer of the plasma mem-
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20 brane is observed in the initial stages of apoptosis. To detect this translocation, we used annexin
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22 V, a protein that binds to cells with exposed phosphatidylserine. A simultaneous cytometric anal-
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24 ysis of annexin V binding and propidium iodide uptake by dead cells was carried out. According
25
26 to Fig. 3A, incubation with 5 μ M P-OGal for 3 h, followed by a 15 min irradiation, leads to about
27
28 25% cell killing. However, no significant change in the percentage of annexin V positive cells
29
30 (Fig. 6A) could be observed up to 9 h following irradiation. Similar results were observed after a
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32 3 h incubation of cells with 1 μ M P-OGal followed by 15 min of irradiation or with 5 μ M P-OGal
33
34 followed by 30 min of irradiation (data not shown).
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41 Another hallmark of apoptosis is nuclear DNA breakdown into oligonucleosomal units of
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43 about 200 bp. The enzyme-linked immunosorbent assay used to quantify the cytoplasmic accu-
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45 mulation of histone-associated DNA fragments shows no significant increase in cytoplasmic his-
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47 tone-associated DNA fragments 0, 1, 3, 6, 9 12 h (Fig. 6B) or 24 h (data not shown) after PDT
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49 with 5 μ M P-OGal.
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53 Activation of intracellular proteases called caspases, is a key event during apoptosis. The cas-
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55 pase cascade is regulated through their partial cleavage. To further examine whether apoptosis is
56
57 involved in the P-R-induced photokilling, caspase-3, caspase-8 and caspase-9 activities have been
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59 assayed. Cleavage of proteins by caspases leads to chromatin condensation and DNA degrada-
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4 tion. Several families of PDT photosensitizers (porphyrins, chlorins, phthalocyanines...) are
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6 strong inducers of apoptosis in various cell lines [12]. Because apoptosis is a dynamic process,
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8 samples have been collected up to 24 h following PDT. No evidence for cleavage of the caspase-
9
10 3, caspase-8 and caspase-9 substrates was found after P-OGal-PDT under the conditions outlined
11
12 above (see Fig. S1 in the supplementary data). The absence of procaspase-3, procaspase-8 and
13
14 procaspase-9 cleavage in Western blots, also shown in the supplementary data, confirms the lack
15
16 of caspase activities. In addition, no cytochrome c leakage has been observed with P-OGal photo-
17
18 sensitized cells (Fig. 7A). To verify that under our experimental conditions, NCTC 2544 cells
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20 may readily undergo apoptosis, such cells were incubated 30 min with 10 μM 5-methoxypsoralen
21
22 in DPBS and then were subjected to UVA irradiation ($2.5 \text{ J}/\text{cm}^2$). Twenty four hours later, NCTC
23
24 2544 treatment with 5-methoxypsoralen+UVA had resulted in extensive activation of caspase-3
25
26 (6-fold increase in activity) and caspase-9 (2-fold increase in activity) in cell lysates. No change
27
28 in activity was observed with caspase-8 (data not shown). These results are fully consistent with
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30 those previously presented by Viola et al. [20]. Another positive control was performed by treat-
31
32 ing cells with 5 μM camptothecin. Measurement of caspase-3 activity and flow cytometry after PI
33
34 and Annexin V staining readily show apoptosis induction only 9 h after treatment. These data
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36 support the conclusion that apoptosis induction after photodynamic injury induced by P-R is in-
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38 significant.
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48 **3.8. Autophagy of NCTC 2544 keratinocytes after PDT with tricationic porphyrins**

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50 Autophagy is a normal process that helps the cell get rid of damaged organelles or macromole-
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52 cules. This highly regulated process is morphologically evidenced by the formation of auto-
53
54 phagosomes and autolysosomes [18, 21]. Recently, a new form of programmed cell death charac-
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56 terized by autophagic hallmarks has been discussed [18, 19]. To determine if programmed cell
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58 death associated with PDT might exhibit such characteristics, we monitored the autophagic activ-
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4 ity in NCTC cells exposed to P-OGal. Autophagic activity was followed using a fluorescent
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6 marker (GFP-LC3) consisting in a fusion microtubule-associated protein light-chain 3 (LC3)
7
8 tagged with a green fluorescent protein (GFP). Upon autophagy induction, LC3 is proteolytically
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10 processed and recruited to autophagosomal membranes [11]. After P-OGal PDT, we have ob-
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12 served the subcellular redistribution of the fluorescent marker GFP-LC3 fusion protein from a
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14 uniform, cytosolic, to a punctated, vacuolar pattern [18, 19] (Fig. 7A). After a 3 h incubation of
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16 cells with 5 μ M P-OGal, followed by 15 min of irradiation, a time-dependent increase in the
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18 number of cells showing the characteristic vacuolar pattern of GFP-LC3 is observed (Fig. 7B).
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20 By contrast, no significant change in the accumulation of GFP-LC3 is observed (Fig. 7B) after
21
22 incubation of NCTC 2544 cells with 0.8 mM ALA for 18 h followed by a 15 min irradiation,
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24 which induces similar photocytotoxic effects as those observed with P-R (see Fig. 3C).
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31 In another series of measurements, the immunoblotting analysis for the modulation of the p62
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33 protein, also called sequestosome 1 (SQSTM1), by P-R has been performed. Since p62/SQSTM1
34
35 accumulates when autophagy is inhibited and decreases when autophagy is induced, it may be
36
37 used as a marker of autophagic activity [18]. Western blotting analysis reveals that 6 h after irradi-
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39 ation the amount of p62/SQSTM1 is decreased by more than 60% in the P-OGal-treated cells but
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41 not in cells treated with P-OGal but not irradiated (Fig. 7D).
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45 To further assess a possible contribution of autophagy to P-OGal-induced cell death, we have
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47 studied the effect of 3-methyladenine (3-MA), a well-characterized autophagy inhibitor [18]. Af-
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49 ter 15 min of irradiation of cells incubated for 3 hours with 5 μ M P-OGal and 5mM 3-MA, the
50
51 percentage of cells showing the characteristic vacuolar pattern of GFP-LC3 in the P-OGal+3-
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53 MA-treated group is decreased to about 70% of the value obtained with the group treated with P-
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55 OGal alone (Fig. 7C). By contrast P-OGal-induced cell death is inhibited by about 40% after in-
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57 cubation with 5mM 3-MA (Fig. 6C). It is well established that the inhibition of autophagy by 3-
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4 MA may potentiate apoptosis induced by a variety of chemicals [21]. Autophagy inhibition does
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6 not significantly change the percentage of annexin V positive cells measured 6 h after P-OGal-
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8 PDT (Fig. 6C). Furthermore, no leakage of mitochondrial cytochrome c in the cytoplasm has
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10 been observed with 3-MA-treated cells up to 6 h after irradiation (data not shown), but the p62
11
12 degradation was decreased by about 80% (Fig. 8D).
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16 These data suggest that cells undergoing P-OGal-PDT show increased autophagy levels and
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18 that autophagy may be associated with cell death induced by P-OGal photosensitization.
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21 **3.9. MAPK participate in death of NCTC 2544 keratinocytes after PDT with tricationic** 22 23 **porphyrins**

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25 Several lines of evidence indicate that MAPK participate in closely related signalling cascades
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27 that contribute to the regulation of gene expression in response to photodynamic treatment. Acti-
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29 vation of ERK 1/2 is generally associated with cell proliferation and survival, while p38 and JNK
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31 are linked to induction of apoptosis [12, 22]. Recent reports show, however, that the role of
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33 MAPKs in cell death induced by photosensitization is largely cell type and photosensitizer-
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35 dependent [12].
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41 The MAPK phosphorylation has been assessed in NCTC 2544 cells treated by P-OGal-PDT
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43 and lysed 0, 1, 3, 6 or 9 h after irradiation. The p38 and JNK phosphorylation was significantly
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45 increased. This increase is stronger with p38 and maximal 3 h after irradiation (Fig. 8A). After
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47 this interval, p38 and JNK phosphorylation steadily declines, becoming barely detectable 9 h af-
48
49 ter irradiation. On the other hand, no significant phosphorylation of p38 and JNK is observed
50
51 before irradiation in untreated (control) cells or after sham-irradiation of P-OGal-treated cells.
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55 Regarding Akt and ERK, the level of total Akt and ERK is unchanged whereas the p-Akt and
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57 p-ERK levels are markedly decreased after P-OGal-PDT in a time dependent manner (Fig. 8A).
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59 In contrast to results obtained with p38 and JNK, substantial amounts of phosphorylated ERK1/2
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4 and Akt are detected prior to irradiation. Although the p-ERK and p-Akt levels remain unchanged
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6 in sham-irradiated cells and untreated controls, p-Akt decreased progressively after the photody-
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8 namic challenge while p-ERK has a significant abrupt drop at 3 hours and becomes barely detect-
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10 able. These results suggest that MAPKs activity modulation by P-OGal-PDT may be implicated
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12 in the cytotoxicity.
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16 To examine the role of MAPKs activation in cell death, P-OGal-treated cells were pre-
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18 incubated with the p38 (SB203580) and JNK (SP600125) inhibitors. As a matter of fact,
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20 SB203580 and SP600125 inhibit p38 and JNK phosphorylation in the NCTC 2544 cells. Thus,
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22 after an osmotic stress induced by 0.4 M sorbitol for 10 min, immunoblot analysis demonstrates
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24 that 25 μ M SB203580 and 25 μ M SP600125 reduced p-p38 and p-JNK over 75% of the control
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26 value (data not shown). Addition of SP600125 3 h before irradiation, increases the photo-induced
27
28 cell death by approximately 50%, whereas SB203580 has no significant effect (Fig. 8B). Inciden-
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30 tally, it must be noted that the viability of control cells treated with P-OGal and pre-incubated
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32 with SB203580 and SP600125 but left in the dark was unchanged. Figure 8B also suggests that
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34 an insignificant effect on the percentage of annexin V positive cells is observed 6 h after P-OGal-
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36 PDT of cells pre-incubated with 25 μ M of p38 and JNK inhibitors. By contrast, 6 h after irradi-
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38 ation, the number of cells showing the vacuolar pattern of GFP-LC3 is increased by 31% ($p <$
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40 0.001) in cells treated with P-OGal and 25 μ M SP600125 (Fig. 8C) although SP600125 has no
41
42 effect on the basal level of GFP-LC3-puncta. On the contrary, no significant change has been
43
44 observed in the P-OGal PDT-induced accumulation of GFP-LC3 after pre-incubation of cells
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46 with SB203580. Under the same experimental conditions, SP600125 but not SB203580 increases
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48 the p62 degradation after PDT (Fig. 8D). Taken together, these results suggest that JNK exerts a
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50 negative regulator effect on P-OGal PDT-induced autophagy and PDT-dependent programmed
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52 cell death.
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4. Discussion

4.1. The nature of the conjugated chain determines the intracellular localization of tri-cationic conjugates and their cytotoxicity.

The comparison of the photocytotoxic potential of P-R is straightforward since they absorb identical light under the same experimental conditions (see supplementary data). The greater photocytotoxic efficiency of P-OGal, P-DDC and P-Me compared to that of P-H and P-Gal (Figs. 2C,D) parallels the magnitude of the lipid peroxidation (Fig. 3A) suggesting that their phototoxicity and their overall ability to produce an oxidative stress correlate, irrespective of the targets in the cell lines studied.

Interestingly, P-OGal, P-DDC and P-Me favorably compare with endogenous PPIX produced by ALA treatment. Figure 3B demonstrates that a 50% loss in Neutral Red uptake is obtained with an intracellular PPIX concentration (~50 pmol/mg of cellular protein) similar to that found for P-OGal, P-DDC and P-Me. Since light doses absorbed by equal concentrations of P-R and PPIX are essentially identical (see supplementary data), P-OGal, P-DDC and P-Me are photosensitizers as powerful as PPIX in our cell model. Comparison of data in Figs. 2B, 3A, 3C demonstrates that at equivalent light doses, oxidative membrane damage is more pronounced with PPIX than with P-Me, P-DDC or P-OGal. This behavior is consistent with the higher hydrophobicity of PPIX favouring its intracellular localisation in lipophilic microenvironments.

With the exception of P-(Lys)_n, P-R derivatives have comparable singlet oxygen formation quantum yield. Consequently, they have similar intrinsic photosensitizing potential. From Fig. 2B, it may be estimated that incubation with P-Me, P-DDC or P-OGal concentrations in the range 0.7-0.8 μM leads to the same P-R intracellular contents as incubations with 5 μM P-H or 1.4-1.6 μM P-Gal. It may be deduced from Figs. 2C,D that these differences cannot account for the ob-

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4 served differences in cytotoxicity.
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6 The intracellular localization may explain different photocytotoxic responses. Microfluorome-
7 try confirms that P-H and P-Gal are taken up less by NCTC 2544 keratinocytes than is P-Me, P-
8 DDC or P-OGal, whose fluorescence is more specifically localized in spots that may correspond
9 to endo- or pinocytotic vesicles, but not to mitochondria and nucleus. The observations reported
10 here demonstrate that the nature of the conjugated side chain is more critical than the electric
11 charge of the porphyrin ring in controlling the uptake and site of localization of the P-R and, con-
12 sequently, their greater photosensitizing efficiency. It may be thought that major direct damage to
13 the mitochondria and the nucleus by $^1\text{O}_2$ produced by P-R is rather unlikely since $^1\text{O}_2$ does not
14 diffuse a great distance from its site of production [16].
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28 **4.2. The photo-induced cell death shows hallmarks of autophagy**

29 Beside demonstrating for the first time the chain structure dependence of the photocytotoxic po-
30 tential of tri-cationic porphyrins, this study provides new information about mechanisms of cell
31 death and MAPK-mediated stress signalling in our PDT model. It has been demonstrated that
32 many PDT photosensitizers including those used to treat skin lesions in clinical practice, e.g.
33 ALA and photofrin[®], activate several pathways leading to apoptosis [12 and references therein].
34 Our results are in sharp contrast with these reports. Consistent with the absence of apoptosis, no
35 annexin V cell staining (Fig. 6A), no oligonucleosome fragmentation (Fig. 6B) and no caspase
36 activation (see supplementary data) are observed after P-OGal-PDT. Among other factors, these
37 results may be related to the non-mitochondrial localization of the most photocytotoxic P-R (Fig.
38 4). The absence of major mitochondrial damage is also suggested by the lack of cytochrome c
39 leakage (Fig. 7A). On the other hand, we show the appearance of a punctate pattern of GFP-LC3
40 and a decrease in the amount of p62 following PDT with P-OGal (Figs. 7A,B,C). Consistently, 3-
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4 methyladenine, a well-known inhibitor of autophagy, inhibits the photocytotoxicity, lowers the
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6 percentage of cells showing punctate GFP-LC3 and decreases p62 degradation (Figs. 6C, 7C and
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8 8D). The incorporation of P-R in lysosomes or pre-lysosomes in the vicinity of the ER and the
9
10 Golgi apparatus might be responsible for this conjunction of pro-autophagic and anti-apoptotic
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12 events.
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16 There are relatively few recent reports on the induction of autophagy by photodynamic thera-
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18 py and most deal with apoptosis-deficient cells or cells whose caspase activity has been inhibited
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20 [12, 23]. We used apoptosis-competent cells in which porphyrin conjugates induced autophagic
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22 cell death in the absence of apoptosis inhibitor. Treatment with the autophagy inhibitor 3-MA is
23
24 not associated with a significant change in the percentage of annexin V positive cells and thus
25
26 with apoptosis activation (Fig. 6C). Possibly, autophagy may constitute an attempt by cells to
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28 remove photo-oxidized organelles or large cross-linked aggregates formed after photodynamic
29
30 damage to protein complexes.
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34 35 **4.3. Consequences of P-OGal-PDT on MAPKs in NCTC 2544 cells**

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37 Several studies performed with different cell lines and photosensitizers have shown that MAPKs
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39 participate in the oxidative signalling events induced by PDT [12]. Previous research presented
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41 conflicting data concerning the roles of p38 and JNK in cell viability after PDT. Results suggest
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43 that the degree of p38 and JNK phosphorylation as well as their effect on cell death regulation
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45 after PDT may depend on cell type, photosensitizer and light dose [22, 24]. In contrast to apop-
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47 tosis, relatively little is known about PDT-induced autophagy signalling in terms of MAPK acti-
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49 vation and possible cross-talk among different pathways.
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54 The role of JNK in cell death has long been controversial as it exhibits a pro-survival function
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56 or it promotes lethality depending on the physiological or pathological condition studied [25].
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58 The paradoxical nature of this effect could be partially due to different modes of JNK activation
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4 [26], different kinetics [27] or might depend on the nature of the chemical inducers used includ-
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6 ing reactive oxygen species [26, 28]. An early transient JNK activation determines cell survival
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8 whereas sustained JNK activation can induce cell death signals [27]. Consistent with these data,
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10 we observed a rapid and transient JNK activation after P-OGal-PDT associated with a negative
11
12 regulatory effect on cell mortality and autophagy (Fig. 8A). We may speculate that this effect
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14 might be partially explained by JNK1 mediated Bcl-2 phosphorylation known to inhibit the bind-
15
16 ing of Bcl-2 to Beclin 1 which activates autophagy [29]. Of note, it has recently been reported
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18 that JNK activation, induced by 2-methoxyestradiol in Ewing sarcoma cells, modulates auto-
19
20 phagy either through Bcl2 phosphorylation or up regulation of the damaged regulated autophagy
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22 modulator [30, 31].
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28 While several reports support the role of p38 as inhibitor of autophagy [32], others imply the
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30 opposite [33]. Although we observed a p38 activation after P-OGal PDT, our data suggest that
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32 p38 is not directly implicated in induction of cell death. A downstream event in the mitogenic
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34 pathway is ERK activation through binding of ligands to extracellular growth factor receptors
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36 involved in regulation of growth and cell cycle progression. The Ras/Raf/ERK activation path-
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38 way can promote opposite pro-survival or anti-proliferative cellular responses, such as apoptosis
39
40 and autophagy. This wide variety of processes triggered by the activation of a single pathway
41
42 depends on the timing, duration and strength of activation, on subcellular localization and on the
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44 presence of reactive oxygen species (ROS) [34]. Available evidence suggests that the photoox-
45
46 idative stress induced by PDT may modulate ERK activity as does other ROS such as H₂O₂,
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48 which is produced in a variety of tumor cell lines by 1,3-dibutyl-2-thiooxo-imidazolidine-4,5-
49
50 dione [35]. Depending on the photosensitizer and cell culture, ERK phosphorylation variably
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52 responds to the PDT-induced oxidative stress by irreversible inhibition [24], moderate attenuation
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54 [36], insignificant modulation [22] or transient activation [37]. It is noteworthy that ALA-PDT
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4 has no effect on ERK expression in HaCat cells [38]. We found a decrease in the p-ERK content
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6 to almost undetectable levels 3 h after after P-OGal-PDT despite high pre-existing expression
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8 (Fig. 8A).
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11 Activation of PI3-K/Akt is another important signaling pathway. The Akt acts on a variety of
12
13 ways including mTOR activation, Bad phosphorylation, I κ B kinase phosphorylation and caspase-
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15 9 inhibition, thereby preventing cell death [21]. The PDT effect on Akt regulation varies from de-
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17 phosphorylation and subsequent activation of caspase-assisted death [39] to phosphorylation
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19 stimulation [40]. Our results show a time-dependent decrease in the content of p-Akt after PDT
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21 treatment (Fig. 8A), consistent with increased photo-induced autophagic death.
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26 **5. Conclusions**

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28 Beside necrosis, autophagy may contribute to the death of NCTC 2544 keratinocytes by P-OGal-
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30 PDT with no evidence for significant apoptosis. JNK activation exerts a negative regulatory ef-
31
32 fect on photocytotoxicity and autophagy while p38 activation is not involved in cell death. Sever-
33
34 al studies show that ERK and PI3-K/Akt pathways are aberrant in human cancers [41] suggesting
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36 them as targets for the development of novel cancer treatments [42]. The inhibition of ERK1/2
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38 and Akt activities suggests that water-soluble tri-cationic porphyrins may thus play a beneficial
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40 role in cancer treatment by PDT given their potentially easy formulation. However, the role of
41
42 MAPKs modulation on photocytotoxicity and autophagic cell death induced by P-OGal-PDT still
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44 needs clarification. In this regard, the use of the siRNA technology would of definite interest to
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46 understand the role played by the JUNK pathway in the photo-induced death mechanism. Finally,
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48 the present study has allowed the characterization of P-OGal as the most potent photoactive con-
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50 jugate in the NCTC 2544 cell model. In view of its potential interest as a PDT photosensitizer in
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52 dermatology, it would also be valuable to assess its photobiological activity towards SCC or BCC
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4 cell lines.

5 6 **Acknowledgements**

7
8
9 This work was supported by an INSERM/GRICES exchange agreement, a Franco-Portuguese
10 "PESSOA" exchange program (07958NF), a co-tutelage "PAUILF" Franco-Portuguese program
11 (J.N.S., P.F., J-C.M., R.S., P.M.) and grants from the Gulbenkian Foundation (J.N.S., P.F.) and
12 the "Fundação para a Ciência e a Tecnologia" (J.P.C.T., M.G.P.M.S.N., E.M.P.S., J.A.S.C.). A
13
14 portion of the epifluorescence microscopy experiments was performed with the equipment of the
15
16 "Centre de Microscopie du Muséum". The authors wish to thank Drs. M. Gèze and M. Dellinger
17
18 for their advices. The authors wish to thank Pr. L.K. Patterson (Radiation Laboratory, University
19
20 of Notre Dame, Notre Dame, Indiana) for his careful reading of the manuscript and improvement
21
22 of the English language. We thank Drs. Yoshimori and Mizshima (Tokyo Metropolitan Institute
23
24 of Medical Science, Japan) for the kind gift of the GFP-LC3 construct.
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Figure Legends

Fig. 1 - Chemical structure of P-R derivatives.

Fig. 2 - A,B: Uptake of P-H, P-Me, P-DDC, P-OGal, P-Gal (●,○,□,◇,■) by NCTC 2544 keratinocytes as a function of the time of incubation with 1 μ M P-R (A) and of the P-R concentration during 3 h incubation (B) in FCS-supplemented culture medium. Data are the mean of at least three independent experiments performed in triplicates. **C,D:** Cytotoxic effects photosensitized by P-H, P-Me, P-DDC, P-OGal, P-Gal (●,○,□,◇,■) as a function of the irradiation time (C) and of the incubation concentration (D) in FCS-supplemented culture medium. In C, cells were incubated for 3 h with 5 μ M P-R prior to irradiation, whereas in D cells were irradiated for 30 min after incubation for 3 with the desired P-R concentration. A 100% Neutral Red (NR) uptake corresponds to sham-irradiated untreated cells. Data are the mean of at least three independent experiments performed in triplicates. Note the logarithmic Y-axis in C.

Fig. 3 - A: Lipid peroxidation photosensitized by P-H, P-Me, P-DDC, P-OGal and P-Gal (●,○,□,◇,■) after incubation for 3 h with 5 μ M P-R in FCS-supplemented culture medium prior to irradiation. TBARS released in the supernatant, normalized to the protein content, are expressed as the percentage of TBARS released after 60 min of irradiation of cells incubated with P-H (100% correspond to 37 ± 7 pmol/mg). Data are the mean of three independent experiments performed in triplicates. **B,C:** Photosensitized cytotoxic effects (B) and lipid peroxidation (C) as a function of the intracellular PPIX concentration after ALA treatment. Cells were treated for 18 h without or with ALA (0.1 to 0.8 mM) in FCS-supplemented culture medium and irradiated for 30 min. A 100% Neutral Red uptake corresponds to sham-irradiated untreated cells. TBARS released in the supernatant were normalized to the protein content. Each symbol corresponds to an independent experiment performed in triplicates. Closed symbols in B and C correspond to data

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4 obtained from the same experiment.

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6 **Fig. 4 - A-R:** Phase contrast images (A-F) and fluorescence micrographs (G-R) obtained with
7 NCTC 2544 keratinocytes incubated for 3 h in EMEM + 10% FCS with no additives (A,G,M),
8 with 5 μ M P-H (B,H,N), P-Me (C,I,O), P-DDC (D,J,P), P-OGal (E,K,Q) or P-Gal (F,L,R). Fluorescence
9 images were obtained with a Nikon \times 100 oil immersion objective. Exposure time: 2 s.
10 Signal attenuation: 1/16. Dichroic mirror: 400 nm. Excitation: 330-380 nm. Emission: 610-700
11 nm. G to L: 256 grey levels between 0 and the maximal fluorescence recorded with P-DDC
12 (show relative intensities). M to R: 256 grey levels between 0 and individual fluorescence (show
13 the topography of localization). White bar = 20 μ m.
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26 **Fig. 5 – Double-labeling experiments: A-C:** Phase contrast images (A) and fluorescence micro-
27 graphs (B,C) obtained after incubation for 3 h with 5 μ M P-DDC in EMEM + 10% FCS and then
28 for 30 min with 5 μ M P-DDC and 125 nM LysoTracker Green in EMEM + 10% FCS. **D-F:**
29 Phase contrast images (D) and fluorescence micrographs (E,F) obtained after incubation for 3 h
30 with 5 μ M P-DDC in EMEM + 10% FCS and then for 30 min with 5 μ M P-DDC and 10 μ M
31 Rhodamine 123 in EMEM + 10% FCS. Objective, signal attenuation, dichroic mirror and excita-
32 tion as in Fig. 4. Exposure times: 2 s with the red emission filter and then 2 s with the green emis-
33 sion filter. Emission: 610-700 nm (B,E) and 510-565 nm (C,F). White bar = 20 μ m.
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45 **Fig. 6 - A:** Flow cytometric analysis of NCTC 2544 keratinocytes after P-OGal photosensitiza-
46 tion. Cells were incubated for 3 h with 5 μ M P-OGal in FCS-supplemented culture medium, then
47 irradiated (I) or sham-irradiated for 15 min (SI). They were stained with Annexin V-FITC and PI
48 and analyzed by flow cytometry at the indicated times after irradiation (I+). Data are the mean of
49 3 independent experiments performed in triplicates. A-/PI-: viable cells; A+/PI-: still viable cells
50 undergoing apoptosis (early apoptotic cells); A+/PI+: late apoptotic dead cells; A-/PI+: necrotic
51 cells. * $p < 0.05$ compared to matching SI. SI data were obtained immediately after sham-
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4 irradiation and were unchanged for durations up to 9 h after sham-irradiation. **B:** After incubation
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6 and irradiation as in (A), cell lysates were prepared and analyzed for cytoplasmic accumulation of
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8 histone-associated DNA fragments by an enzyme-linked immunosorbent assay at the indicated
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10 times after irradiation. CT: sham-treated control cells; P-OGal (SI): treated cells but sham-
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12 irradiated for 15 min; P-OGal (I) cells treated and irradiated for 15 min. Data are the mean of 3
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14 independent experiments performed in triplicates. ** $p < 0.01$ compared to matching CT. **C:** Ef-
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16 fect of 3-MA on cytotoxic effects photosensitized by P-OGal. Before irradiation, cells were
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18 treated for 3 h with 5 μM P-OGal only (w/o 3-MA) or simultaneously with 5 μM P-OGal and 5
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20 mM 3-MA. Cells were stained with Annexin V-FITC and PI 6 h after irradiation and analyzed by
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22 flow cytometry. Data are the mean of 3 independent experiments performed in triplicates. * $p <$
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24 0.05 and ** $p < 0.01$ compared to 3-MA untreated cells.
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31 **Fig. 7 - P-OGal induced autophagic cell death in NCTC 2544 keratinocytes. A:** Cells transfected
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33 with GFP-LC3 (green) were treated for 3 hours with 5 μM P-OGal and then irradiated for 15 min.
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35 Fluorescence micrographs were simultaneously recorded after cytochrome c immunofluorescent
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37 staining (red) and nucleus staining with 4,6-diamidino-2-phenylindole (DAPI; blue). Images are
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39 representative of cells with 3 or more puncta of GFP-LC3. Whereas untreated healthy cells (Con-
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41 trol) show diffuse distribution of GFP-LC3, 6 h after irradiation, P-OGal treatment (Irradiated)
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43 induces GFP-LC3 puncta indicating the formation of autophagosomes. Transfected cells treated
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45 with 0.8mM ALA for 18 h and irradiated for 15 min give essentially the same puncta distribution
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47 as untreated cells (not shown). Simultaneously, unirradiated healthy cells display a cytochrome c
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49 perinuclear rod-like pattern consistent with location in mitochondria. A punctate pattern with no
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51 diffuse cytoplasmic distribution of fluorescence is observed in irradiated cells suggesting that
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53 cytochrome c remains in mitochondria. White bar = 20 μm . **B:** Transfected cells treated with P-
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55 OGal or ALA presenting 3 or more GFP-LC3 vesicles were counted at indicated times after irradi-
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4 iation (I+). CT: controls (sham-treated sham-irradiated cells); SI: cells treated with P-OGal or
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6 ALA but sham-irradiated. Results from 3 independent experiments in triplicates are given as a
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8 percentage of autophagic cells over 500 transfected cells (mean \pm SD). ** $p < 0.01$ compared to
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10 matching CT. SI data were obtained immediately after irradiation and were unchanged up to 6 h
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12 after sham-irradiation. Sham-treated irradiated cells behave as CT. **C:** Cells transfected with
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14 GFP-LC3 were treated with P-OGal or ALA and irradiated as described in A, without or with
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16 simultaneous treatment with 5 mM 3-MA. Autophagic cells were counted and results are given as
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18 in B (mean \pm SD of 3 independent experiments in triplicates). ** $p < 0.01$ compared to matching
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20 3-MA-untreated cells. **D:** Western blotting analysis of lysates from cells collected at indicated
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22 times after treatment with P-OGal and irradiation. Total cell lysates were analyzed for p62. β -
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24 actin is shown as loading control. SI data were obtained immediately after sham-irradiation and
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26 were unchanged up to 9 h after sham-irradiation. Sham-treated irradiated cells behave as CT.

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33 **Fig. 8** - MAPKs protein expressions were analyzed in lysates of NCTC 2544 cells. **A.** Represent-
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35 ative Western blots of total and phosphorylated JNK, p38, ERK and Akt either in cells untreated
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37 but left in supplemented culture medium for 3 hours (control, CT) or in cells treated during 3
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39 hours with 5 μ M P-OGal and then sham-irradiated (SI) or irradiated for 15 min and incubated for
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41 indicated times after irradiation (I+). Total- JNK, p38, ERK and Akt immunoblottings are used as
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43 controls for protein loading. **B.** Effect of 25 μ M SB203580 or 25 μ M SP600125 on cytotoxic
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45 effects photosensitized by P-OGal. Before irradiation, cells were treated for 3 h with 5 μ M P-
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47 OGal (CT) or with 5 μ M P-OGal and 25 μ M SB203580 (SB) or SP600125 (SP). Nine hours after
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49 irradiation, cells were stained with annexin V-FITC and PI and analyzed by flow cytometry. Data
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51 are the mean of 3 independent experiments performed in triplicates. * $p < 0.05$ and ** $p < 0.01$
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53 compared to irradiated CT. **C.** Cells transfected with GFP-LC3 were treated using P-OGal with-
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55 out (CT) or by pre-incubation with 25 μ M SB203580 (SB) or SP600125 (SP). Autophagic cells
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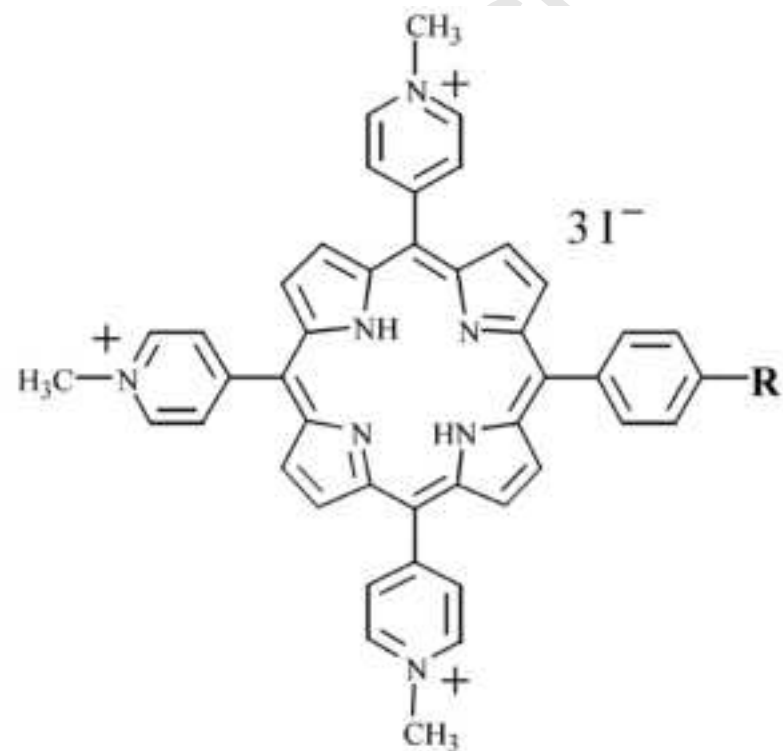
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4 were counted and results are given as in Fig 7B (mean \pm SD of 3 independent experiments in
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6 triplicates). ** $p < 0.01$ compared to matching sham-irradiated cells. **D.** Western blotting analysis
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8 of p62 in lysates from cells collected 9 h after P-OGal-PDT without or with pre-incubation in the
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10 presence of 5 mM 3-MA (3-MA), 25 μ M SB203580 (SB) or SP600125 (SP). β -actin is shown as
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Table 1. Photosensitization of NCTC 2544 keratinocytes by tricationic porphyrins. Photocytotoxic concentration (PC_{50}), intracellular phototoxic content (IPC_{50}) and inhibition of photocytotoxicity by N_3^- ions.

	P-H	P-Me	P-DDC	P-OGal	P-Gal	P-(Lys) _n ^c
PC_{50} (μ M) ^a	>> 5	~1.8	~1.5	~1.2	>5	~3.1
IPC_{50} (pmol/mg protein) ^b	>> 35	~55	~55	~50	> 50	~260
Inhibition by N_3^- (%) ^d	100 \pm 7	95 \pm 11	88 \pm 12	93 \pm 12	100 \pm 2	-

^a PC_{50} is defined as the incubation concentration necessary to obtain a 50% decrease in NR uptake after a 30 min irradiation. Data were obtained from Fig. 3B. ^b IPC_{50} is the intracellular phototoxic content corresponding to the PC_{50} obtained from Fig. 2B. ^cData obtained from Silva et al. [5]. ^d N_3^- ions at a non toxic concentration of 20 mM were present during the 3 h incubation with P-R and the subsequent 30 min irradiation. Data represent survival, where 100% corresponds to survival in untreated samples.

Figure 1



	P-H	P-Me	P-DDC	P-OGal	P-Gal	P-(Lys) _n
R						

Figure 2

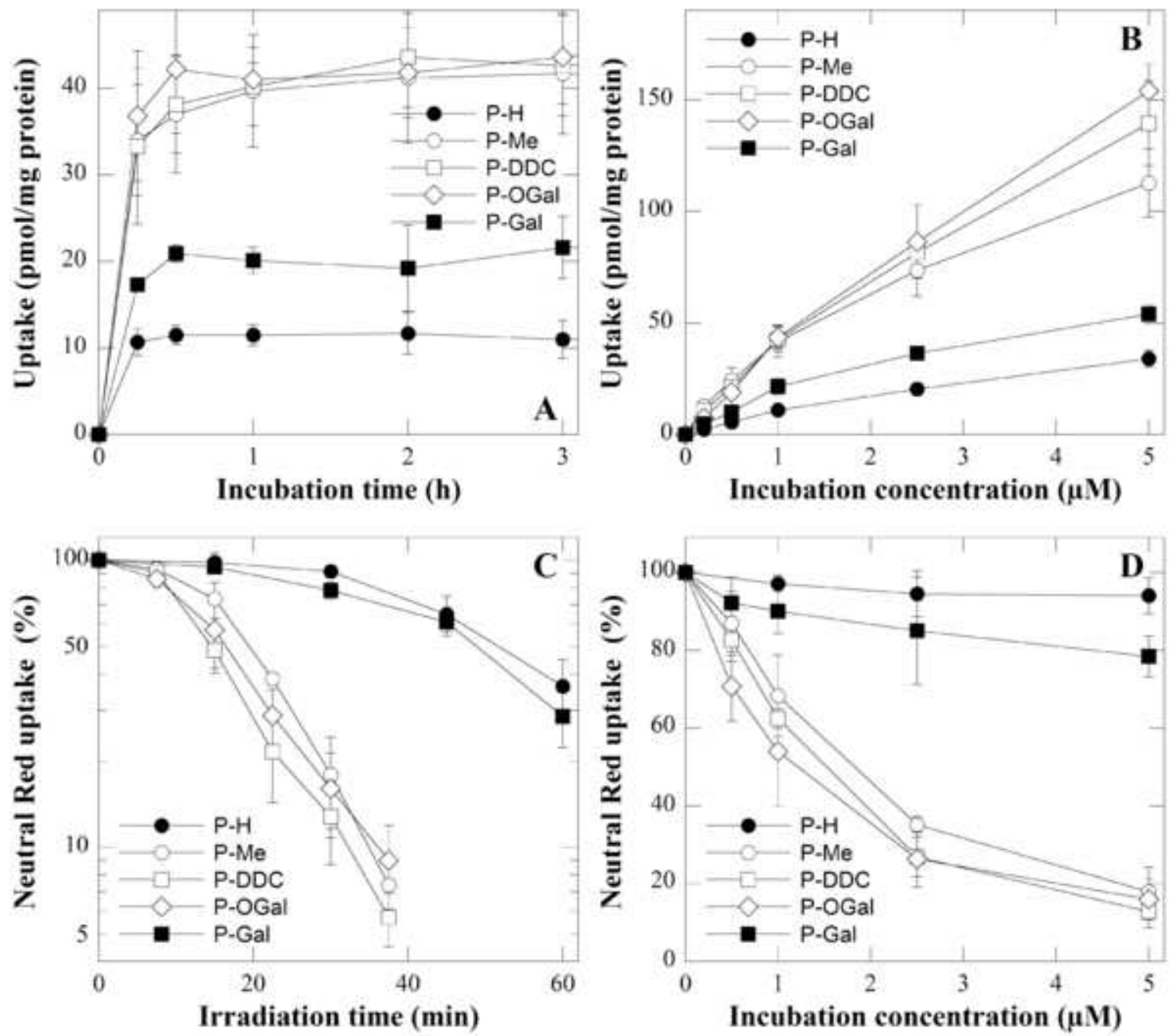


Figure3

