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Liposomal temocene (*m*-THPPo) photodynamic treatment induces cell death by mitochondria-independent apoptosis

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ABSTRACT

Background: The cell death pathway activated after photodynamic therapy (PDT) is controlled by a variety of parameters including the chemical structure of the photosensitizer, its subcellular localization, and the photodynamic damage induced. The present study aims to characterize a suitable *m*-THPPo liposomal formulation, to determine its subcellular localization in HeLa cells and to establish the cell death mechanisms that are activated after photodynamic treatments.
Methods: Liposomes containing *m*-THPPo were prepared from a mixture of DPPC and DMPG at a 9:1 molar ratio. In order to procure the best encapsulation efficiency, the *m*-THPPo/lipid molar ratio was considered. HeLa cells were incubated with liposomal *m*-THPPo and the subcellular localization of *m*-THPPo was studied. Several assays such as TUNEL, annexin V/propidium iodide and Hoechst-33258 staining were performed after photodynamic treatments. The apoptotic initiation was assessed by cytochrome c and caspase-2 immunofluorescence.
Results: *m*-THPPo encapsulated in liposomes showed a decrease of the fluorescence and singlet oxygen quantum yields, compared to those of *m*-THPPo dissolved in tetrahydrofuran. Liposomal *m*-THPPo showed colocalization with LysoTracker® and it induced photoinactivation of HeLa cells by an apoptotic mechanism. In apoptotic cells no relocalization of cytochrome c could be detected, but caspase-2 was positive immediately after photosensitizing treatments.
Conclusions: Photodynamic treatment with liposomal *m*-THPPo leads to a significant percentage of apoptotic morphology of HeLa cells. The activation of caspase-2, without the relocalization of cytochrome c, indicates a mitochondrial-independent apoptotic mechanism.
General significance: These results provide a better understanding of the cell death mechanism induced after liposomal *m*-THPPo photodynamic treatment.

1. Introduction

Photodynamic therapy (PDT) is a clinically approved therapeutic modality for the treatment of neoplastic and non-malignant diseases. It is based on the selective accumulation of a photosensitizer (PS) within the tumor tissue and/or its vasculature, followed by irradiation with visible light. The combination of light and PS, together with the molecular oxygen, induces the formation of reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$), that cause the selective destruction of the irradiated tumor tissue [1–4].

Photodynamic treatment can lead to cell death by four main pathways: necrosis, apoptosis, autophagy and mitotic catastrophe [5–7]. Several parameters, such as the treatment dose, the subcellular localization of the PS or the type of cell line, have been characterized as responsible factors for the different cell death processes [8,9]. Particularly, the subcellular localization of the PS plays an important role in determining the photodynamic efficacy, since $^1O_2$ has a very short lifetime, and therefore, the internalized PS must be located near its intracellular target [10]. A PS can localize in different cellular compartments such as mitochondria, lysosomes, plasma membrane, endoplasmic reticulum, or Golgi apparatus [9,11,12], depending on multiple factors including the chemical structure and its aggregation state, the incubation time and the carrier vehicle [8,13,14].

The apoptotic process can be initiated by various mechanisms that produce different cellular responses depending on the origin of the

Abbreviations: DMPG, dimyristoylphosphatidylglycerol; DMSO, dimethylsulfoxide; DPPC, dipalmitoylphosphatidylcholine; Hoechst-33258, H-33258; *m*-THPPo, m-tetra (hydroxyphenyl) porphycene (temocene); MLVs, multilamellar vesicles; PDT, photodynamic therapy; PI, propidium iodide; PS, photosensitizer; ROS, reactive oxygen species; TB, toluidine blue; THF, tetrahydrofuran; PCS, photon correlation spectroscopy; PBS, phosphate buffered saline

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triggering signal. Apoptotic cell death can be induced by extracellular (extrinsic apoptosis) or intracellular signals (intrinsic apoptosis). It is generally accepted that mitochondria plays a major role in triggering PDT-induced apoptosis [7], but increasing evidences suggest that other organelles, such as lysosomes, Golgi apparatus or endoplasmic reticulum are also important points of integration for pro-apoptotic signaling and damage sensing [15–17]. Several molecular events have been associated with apoptosis induced by different organelles, but usually they converge in a common pathway [18,19]. Thus, similar cell morphologies can result from different molecular processes.

The novel PS temocene (m-tetra(hydroxyphenyl) porphycene; *m*-THPPo) was chosen as the sensitizing molecule for this study. In a previous report [20], we demonstrated its efficacy on HeLa cells when temocene was dissolved in dimethylsulfoxide (DMSO). However, its high hydrophobicity and easy aggregation in aqueous environment made it necessary to develop a drug delivery system that could incorporate the PS in monomeric form. Liposomes have been one of the most intensively studied drug delivery systems for PSs. These lipid nanoparticles possess several features required for an effective vehicle, such as biocompatibility, suitability for carrying hydrophilic or hydrophobic molecules and the possibility to easily change its size, charge and surface properties [21,22].

2. Materials and methods

2.1. Preparation of liposome lyophilizates

The synthesis and molecular characterization of *m*-THPPo have been previously described in detail [20]. Liposomes were prepared by microemulsification. Dipalmitoylphosphatidylcholine and dimyristoylphosphatidylglycerol sodium salt (DPPC and DMPG respectively) lipid mixtures at the 9:1 molar ratio containing *m*-THPPo were evaporated to dryness from a chloroform solution and kept in a vacuum desiccator for 12 h over P_2O_5 in order to remove the last traces of the solvent. Multilamellar vesicles (MLVs) were prepared by hydration of the dried lipid film by vortexing for 30 min (alternating 30 s periods of heating and 30 s of vortexing) at a concentration of 10 mg lipid/mL of 10 mM PBS buffer (pH 7.4) at 60 °C. The MLV dispersion was frozen and thawed (five times), sonicated (bath sonicator, 15 min, 60 °C) and microemulsified (EmulsiFlex B3 device, Avestin, Ottawa, Canada). Microemulsification was carried out by pumping the fluid fifteen times through the interaction chamber (60 °C, 200 kPa). The liposomes were stored in the dark at 4 °C. Subsequent liposome handling procedures were all performed in the dark. To enhance stability during storage, the final liposomal formulation was lyophilized using 5% trehalose as cryoprotectant agent. Two milliliters of liposomal suspension was placed in 4 mL glass vials and frozen at −80 °C (liquid nitrogen) during 3–5 h. Vials were subsequently dried during 24 h at −55 °C and 0.04 mbar (Freeze Dryer Alpha 1–2/LD, Martin Christ GmbH, Osterode, Germany). Lyophilized liposomes were rehydrated immediately before the experiments by adding 2 mL of sterile water. The suspension was prewarmed at 60 °C during 15 min and vortexed for 30 min (alternating 30 s periods of heating/vortexing). For cellular experiments, the rehydrated liposome suspension was diluted in serum-free medium to 10 μM *m*-THPPo and sterilized by successive filtration through 0.45 μm (Pall Corporation, New York, NY) and 0.22 μm (Millipore®, Bedford, MA) filters. Finally, the appropriate working concentration was achieved by diluting the processed sample with serum-free medium.

2.2. Liposomes characterization

The PS content of all formulations was evaluated following standard procedures. Liposomes were disrupted by the addition of DMSO to an aliquot of the liposomal suspension, free of the non-entrapped PS, and the absorbance was measured at λ_{max} of the Soret band (Cary 6000i UV–vis–NIR spectrophotometer, Agilent Technologies, Santa Clara, CA; or Shimadzu UV-1601 spectrophotometer, Shimadzu Scientific Instruments, Kyoto, Japan). The PS concentration was calculated by comparison with standard curves at different concentrations ranging from 1 to 10 μM (correlation coefficient 0.999) obtained under the same conditions. Lipid content was quantified by a colorimetric assay with ammonium ferrothiocyanate according to the method of Stewart [23]. Briefly, an aliquot of 10 μL of liposomes was disrupted with 2 mL of chloroform and mixed with 2 mL of 0.1 M ammonium thiocyanate. After vortex shaking for 1 min, the sample was centrifuged for 5 min at 4000 rpm. The absorbance of the reddish organic phase is read at 470 nm. The phospholipid concentration is determined by comparison to the appropriate calibration curve obtained with known amounts of phospholipid and the zeta potential was determined by PCS (Malvern Zetasizer NANO-ZS device, equipped with an optic unit containing a 5 mW He–Ne laser, Spectra Physics, and an electrophoresis cell, Malvern Instruments Ltd, Malvern, UK). Before measuring, samples were appropriately diluted to avoid multiple scattering. To evaluate the aggregation state, the photophysical properties of *m*-THPPo encapsulated in liposomes were measured. Absorption spectra were recorded in a Cary 6000i UV–vis–NIR spectrophotometer (Agilent Technologies). Fluorescence emission spectra were recorded in a Fluoromax-4 spectrofluorometer (Horiba Jobin-Yvon, Edison, NJ). The fluorescence quantum yield (Φ_F) was determined from the comparison of the area under the corrected emission curves of optically-matched solutions of the sample to that of a reference, in this case, cresyl violet. Fluorescence decays were recorded in a time-correlated single photon counting system PicoQuant Fluotime 200 (PicoQuant GmbH, Berlin, Germany) with a 375-nm excitation LED. The fluorescence decays were analyzed using the PicoQuant FluoFit 4.0 data analysis software. Singlet oxygen phosphorescence was detected using a customized PicoQuant Fluotime 200 described elsewhere [24]. Singlet oxygen quantum yield (Φ_Δ) was determined from the comparison of the phosphorescence signal amplitudes of optically-matched solutions of the sample to that of a reference, in this case, 5,10,15,20-tetrakis (N-methyl-4-pyridylium)-21*H*,23*H*-porphine (TMPyP) ($\Phi_{\Delta,\ H2O} = 0.74$, [25]).

2.3. Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/mL streptomycin and 1% (v/v) 0.2 M L-glutamine. All products were provided by Gibco (Paisley, UK). Cell cultures were performed in a 5% CO_2 atmosphere at 37 °C and maintained in a Steri-Cult 200 incubator (Hucoa-Erloss, Madrid, Spain). The cells were seeded on 24-multiwell dishes (Falcon, 9 × 14 cm) with or without coverslips. Experiments were performed with cells at 60–70% of confluence.

2.4. Photodynamic treatments

HeLa cells were incubated in serum-free medium with 0.5, 1, 2 and 5 μM *m*-THPPo encapsulated in liposomes for 24 h. Occasionally and for comparative purposes, cells were incubated for 24 h with 2 μM *m*-THPPo directly dissolved in DMSO [20]. The final concentration of DMSO was less than 0.1% and it did not affect the cell survival. After treatments, cells were washed three times with fresh PBS and maintained in whole medium during irradiation and post-treatment. Red light irradiation was performed using a LEDs source ($\lambda = 640 \pm 20$ nm) with a mean intensity of 4 mW/cm^2, measured with an M8 Spectrum Power Energy-meter. The light dose delivered in all experiments was 3.5 J/cm^2. The device is composed by 384 LEDs arranged in an area of 11 cm × 15 cm.