Comparative Antiviral (HIV) Photoactivity of Metalized *meso*-Tetraphenylsulfonated Porphyrins

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Abstract: We have carried out the study of the photochemical properties of a series of synthetic *meso*-tetraphenylsulfonated porphyrins ($TPPMS_4$) bonded to several metal ions such as: Cu(II), Zn(II), Pd(II), Mn(II), Fe(III), Ni(II) and Co(II) for the optimization of their clinical applications as antiviral agents against the human immunodeficiency virus (HIV-1) as well as the study of the *in vitro* antiviral photoinactivation mechanisms with future application in blood sterilization. A selective inhibition has been determined in the viral growth (HIV-1) when this is irradiated in the presence of the complex $TPPFeS_4$ and $TPPMnS_4$ (photosensitizer-mediated Type I reaction) as well as in the $^1O_2$-mediated (Type II reaction) in the presence of $TPPPdS_4$ and $TPPZnS_4$, remaining cellular viability unaltered in each case.

Key Words: *meso*-Tetraphenylsulfonated porphyrins, HIV, metallo porphyrins, photoinactivation, phototherapy, phototoxicity, photosensitizer, antiviral, singlet oxygen.

Dedicated to: Dr. Carlos Rivas's 74th birthday

INTRODUCTION

Photochemical studies on new photosensitizers for photodynamic therapy (PDT), such as Pheophytin, Radachlorin, Mg-chlorophyll-a and -b, as well as other natural compounds have been carried out in our laboratory [1-4]. The most extensively studied photosensitizers are porphyrins that were identified over 150 years ago. Two of their good features are minimal toxicity in the dark and a lack of pharmacological interactions with other drugs, making PDT a safe procedure in oncological combined treatments (either PDT and drug or radiation). For a long time most preclinical studies were dominated by the use of hematoporphyrin derivatives. The best clinical experience has been obtained with the use of Photofrin which is a mixture of monomers, dimers and oligomers derived from hematoporphyrin. As it is very well-known singlet oxygen is a reactive species of oxygen able to promote harmful processes at cellular level, such as: lipid peroxidation, damage of cellular membrane and cellular death. The photosensitizers in our study are usually derived from porphyrins and chlorophylls, originating high yields of reactive oxygen species (ROS) such as $^1O_2$, $^{\cdot}OH$ causing *in vitro* phototoxicity. Some of them have been exhaustibly studied in pre-clinical investigations, showing significant advantages over other procedures used in dynamic anti-viral and anti-tumoral phototherapy [5-9]. The PDT is a novel treatment for cancer and other abnormal tissue degeneration processes that employs a photosensitizer and visible light to produce singlet oxygen and other reactive oxygen species that lead to subcellular damage at the sites where the photosensitizer accumulates. Alternatively, an excited photosensitizer may react directly with biomolecules to form free radicals that further react with molecular oxygen producing the superoxide radical anion, hydrogen peroxide or hydroxyl radical. The superoxide radical anion for instance is generated by excitation of porphyrins in the presence of reducing substances. The objective of this study was to investigate the ability of Cu(II), Zn(II), Pd(II), Mn(II), Fe(III), Ni(II) and Co(II) complexes of *meso*-tetraphenylsulfonated porphyrins ($TPPMS_4$), Fig. (**1**), to generate the reactive oxygen species ($^{\cdot}OH$, $^1O_2$) upon irradiation with UVA-Vis light [10]. The ability of these compounds to generated $^{\cdot}OH$ in cell-free systems in the presence of luminol chemiluminescence was

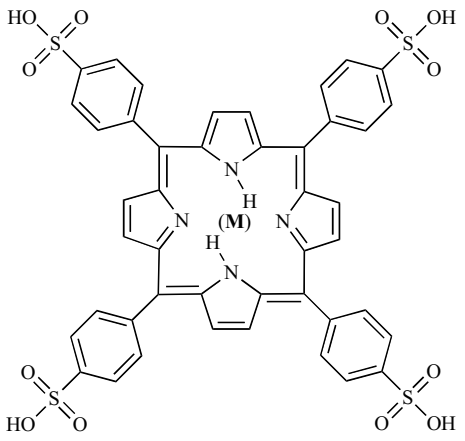

Fig. (1). Structure of $TPPMS_4$ studied, M = Cu, Zn, Pd, Mn, Fe, Ni, Co.

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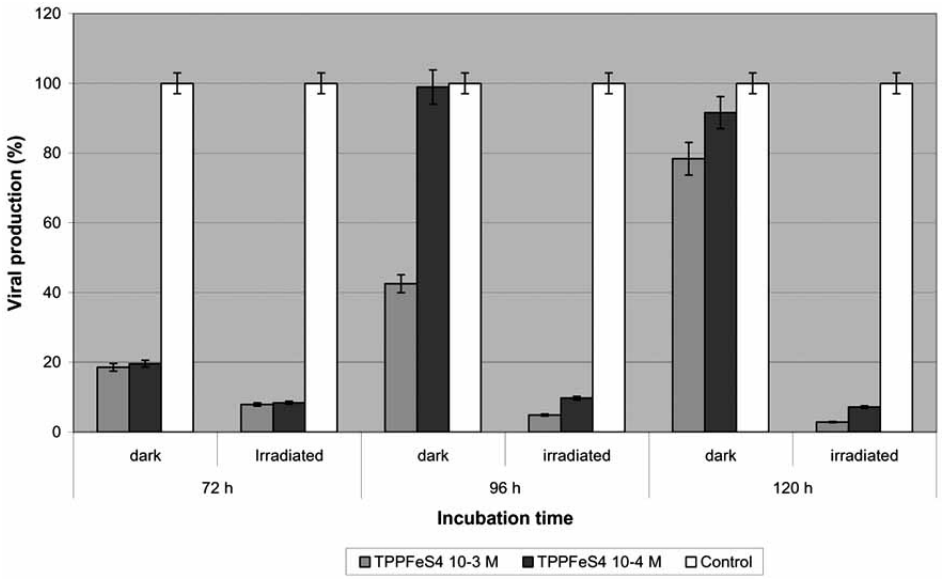

Fig. (2). Effects of $TPPFeS_4$ on the viral production (HIV-1) under irradiated and dark conditions by Elisa assay.

in vestigated. These compounds are also capable of producing singlet oxygen by energy transfer when they are irradiated with UV-A and visible light in the presence of molecular oxygen. We have investigated the optimization of their clinical applications as antiviral agents against the human immunodeficiency virus (HIV-1) and the blood sterilization by means of *in vitro* viability and as well as the ELISA assay for evaluation purposes.

RESULTS AND DISCUSSION

Preparation and Determination of the Metalized Porphyrin Complexes

Absorption, emission, ^{1}H-, ^{13}C-NMR, EPR spectra of the sulfonated metalloporphyrin complexes, was compared with theses authentic or similar parent compounds in the literature [11-16]. All the metalized porphyrins contain divalent metal, except for Fe, which was determined to have valence III with Cl as contra-ion.

Formation of ROS

The generation of ·OH was determined by means of luminol chemiluminescence after the irradiation (λ= 400-900 nm) of the metallic of *meso*-tetraphenylsulfonated porphyrins complexes of Cu(II), Zn(II), Pd(II), Mn(II), Fe(III), Ni(II) and Co(II) in presence of NADPH (data not shown). The formation of peroxidic species that activate the luminol luminescence showed the following tendency: $TPPPdS_4$ > $TPPFeS_4$ > $TPPMnS_4$ > $TPPZnS_4$ > $TPPCuS_4$ > $TPPS_4$ > $TPPNiS_4$ > $TPPCoS_4$.

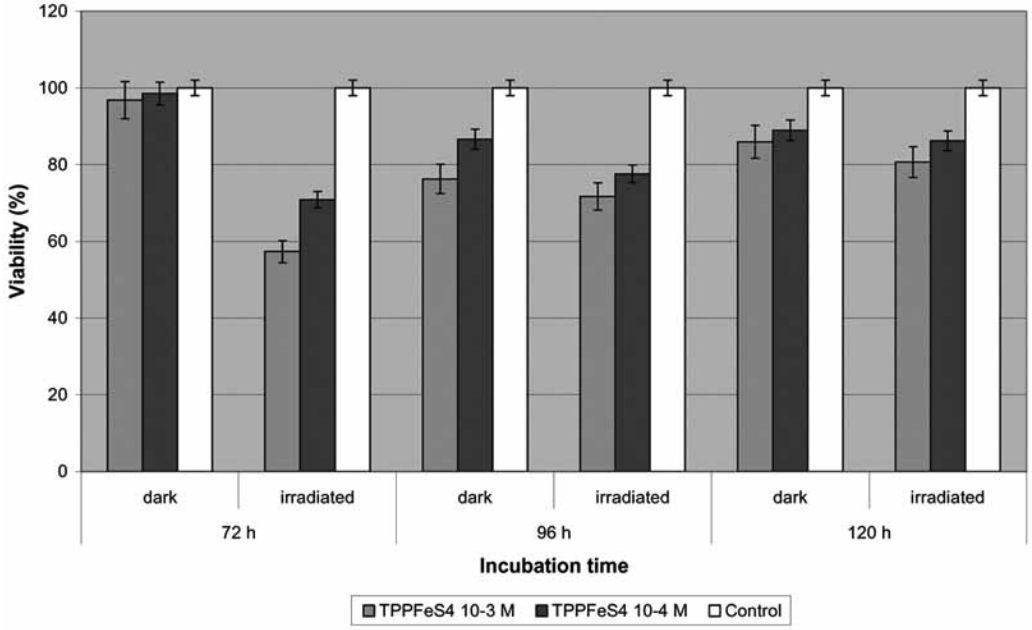

Fig. (3). Effects of $TPPFeS_4$ on the cellular viability under irradiated and dark conditions as determined by a viability assay.

The detection of $^1O_2$ was determined by the test of histidine and p-nitrosodimethylaniline relative to Rose Bengal. The best yields in generation of $^1O_2$ were those of $ZnTPPS_4$> $PdTPPS_4$> $CuTPPS_4$. The quantum yields for singlet oxygen generation were estimated in relation to Rose bengal (0.79), $TPPZnS_4$ (0.37), $TPPPdS_4$ (0.14), $TPPCuS_4$ (0.12), $TPPMnS_4$ (0.08), $TPPS_4$ (0.07), $TPPCoS_4$ (0.06), $TPPFeS_4$ (0.04), $TPPNiS_4$ (0.03) which were corroborated by data reported in the literature [17].

Antiviral Photoactivity

The cytotoxicity (MTT-CellTiter96 non radiative cell proliferation assay) with and without light, of MT4 cells free virus remained on 70% of cellular viability in the presence of 10^{-3}, 10^{-4} and 10^{-5} M concentrations of metalized porphyrins. The quantification of these results was carried out by means of citotoxicity and ELISA assays. The inactivation of viral proliferation in HIV infected cells was effective depending on the $TPPMS_4$ and as a consequence of its mechanism of action.

The following graphs show the antiviral activity of the complexes $TPPFeS_4$, $TPPMnS_4$, $TPPPdS_4$ and $TPPZnS_4$ on the production of HIV-1. It is important to emphasize on the fact that cellular viability remains during the irradiation process. The other metallic complexes such as $TPPCuS_4$, $TPPNiS_4$ and $TPPCoS_4$ didn't show appreciable antiviral action when they were irradiated in the presence of cultivated HIV-1.

We can observe in the previous graph that the photoinduced antiviral activity of the Iron complex is remarkable, with a viral annihilation of approximately 80-85% after 120 hours of irradiation, while the toxicity in the darkness is of 10%. On the other hand the cellular viability (normal cells, Fig. **3**) remains almost unaffected (only 10%). This indicates a specific photochemically induced antiviral action against the HIV-1 virus showing a very low toxicity to human cells.

On the other hand, a very high proportion of photoinduced antiviral activity of the Mn porphyrin complexes, almost 98%, was observed (Fig. **4**). The toxicity in the darkness was of 10 to 20%. On the other hand the cellular viability (normal cells, Fig. **5**) remains almost unaffected (20%).

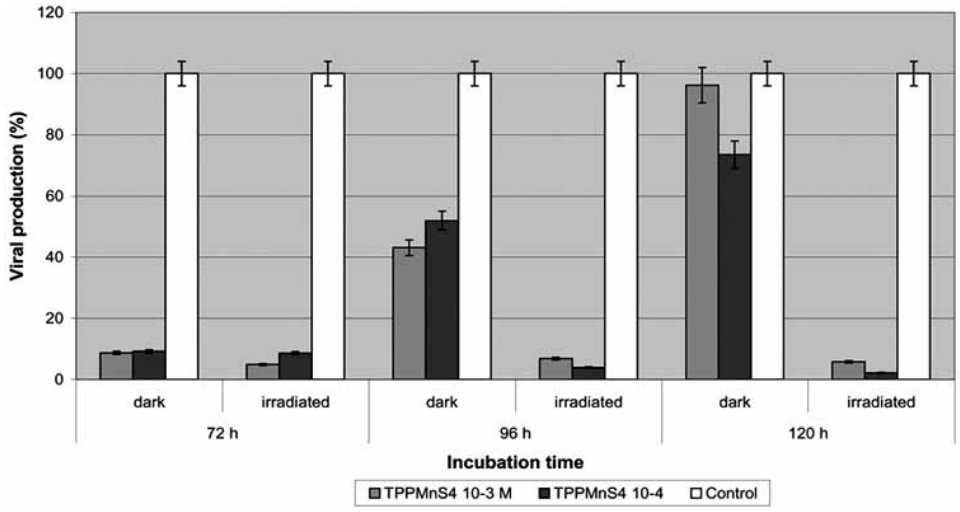

Fig. (4). Effects of $TPPMnS_4$ on the viral production (HIV-1) under irradiated and dark conditions as determined by Elisa assay.

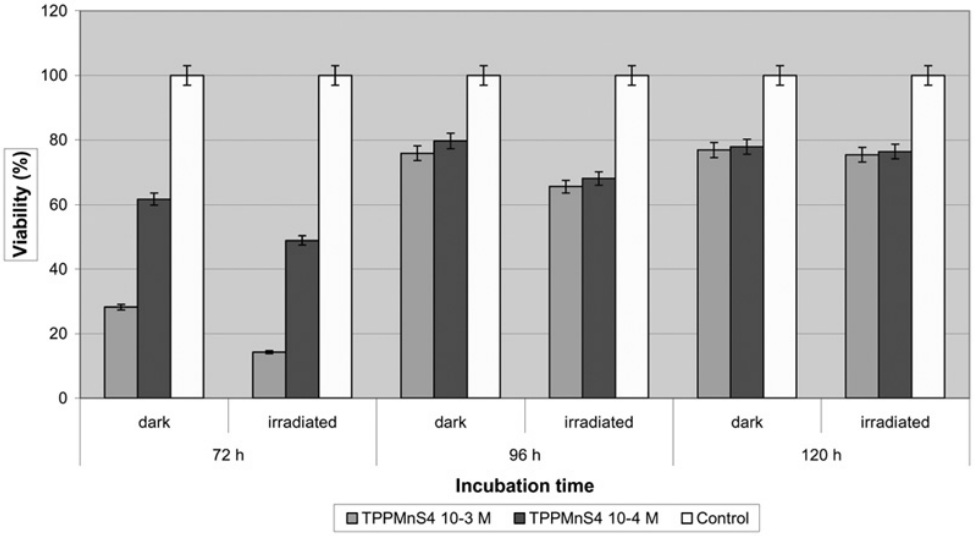

Fig. (5). Effects of $TPPMnS_4$ on the cellular viability under irradiated and dark conditions as determined by a viability assay.

In a same way the complexes of Pd and Zn $TPPS_4$ turned out to have photoactivity against HIV-1. These results are shown in the following Figs. **6-9**.

CONCLUSIONS

Our results suggest that, by means of our experiments, on sensitizer-mediated (type I) reactions as determined for the compounds $TPPFeS_4$, $TPPPdS_4$, $TPPMnS_4$ and $TPPZnS_4$ play an important role in the viral inactivation. On the other hand, compounds such as $TPPPdS_4$ and $TPPZnS_4$ with a high quantum yield for singlet oxygen generation, indicate that $^1O_2$-mediated - type II reactions may also play an important role, in addition to the type I reaction, in the inactivation of cells infected with HIV. However, we can also observe from our results that a compound such as $TPPCuS_4$ with an appreciable generation of singlet oxygen, didn't produce any significant effect in the viral inactivation. On the other hand, complexes of Mn and Fe with a comparative low quantum yield of $^1O_2$ generation, but high formation of peroxidic species *via* type I reaction, produced a significant photoinduced antiviral activity. By either of the two mechanisms, charge (type I reaction) or energy (type II reaction) enough energy is transferred to a substrate or to molecular oxygen to generate reactive oxygen species. Therefore, it has been shown that both types of reaction mechanisms (Types I and II) play an important combination of antiviral action.

A selective inhibition has been determined in the viral growth (HIV-1) when this is irradiated with any of the followin g complexes $TPPFeS_4$, $TPPMnS_4$, $TPPPdS_4$ and $TPPZnS_4$, remaining cellular viability unaltered.

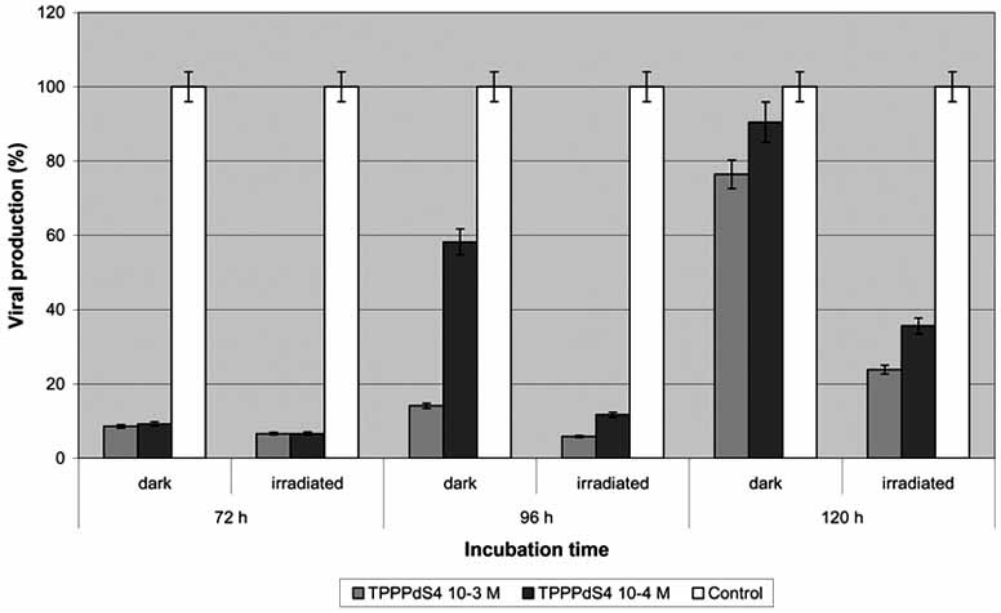

Fig. (6). Effects of $TPPPdS_4$ on the viral production (HIV-1) under irradiated and dark conditions as determined by Elisa assay.

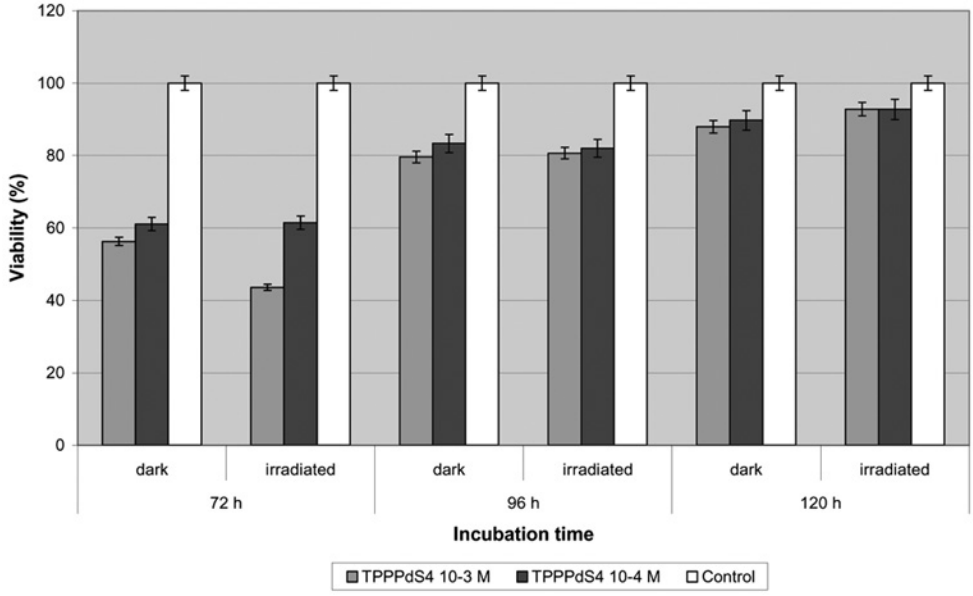

Fig. (7). Effects of $TPPPdS_4$ on the cellular viability under irradiated and dark conditions as determined by a viability assay.

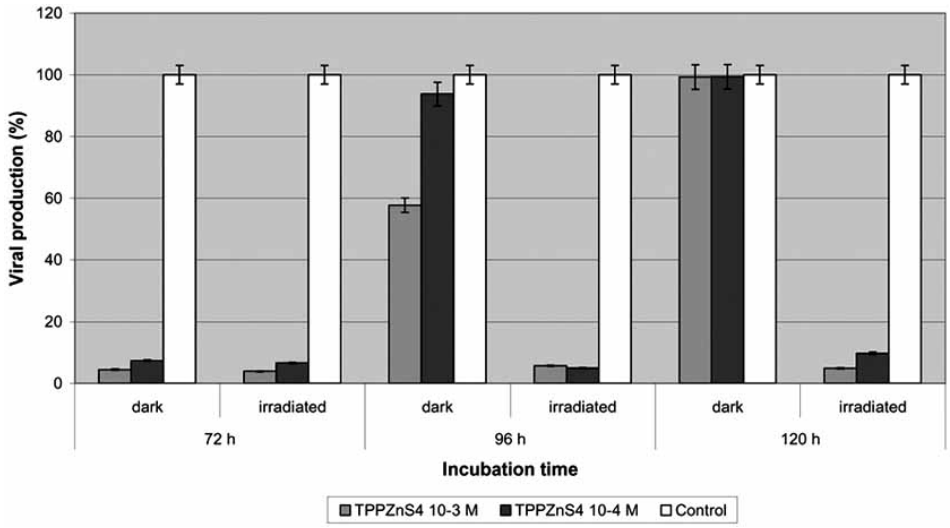

Fig. (8). Effects of $TPPZnS_4$ on the viral production (HIV-1) under irradiated and dark conditions as determined by Elisa assay.

Low viral productions but high viability of infected cells is critical. $TPPFeS_4$ > $TPPMnS_4$ > $TPPZnS_4$ > $TPPPdS_4$ >>> $TPPCuS_4$, $TPPNiS_4$ and $TPPCoS_4$. The results demonstrate that the anti-HIV activity of the sulfonated tetraphenyl metalized porphyrins is relatively independent of the nature of the central metal atom. Indicating that a range of structure may be effective for viral inhibition. An apparent relationship exists among the feasibility of producing bigger quantity of ROS and a better anti-HIV activity. For example, sulfonated tetranaphthyl porphyrins ($TNapS_4$) and their metalized compounds following another order in relation to the effectiveness viral inactivation: $TNapCuS_4$ > $TNapS_4$ > $TNapFeS_4$ [2].

The phototoxic character of the photosensitizer compounds with an efficient generation of singlet oxygen is today an important topic of investigation, specially when it is related to other viruses in addition to HIV such as vesicular estomatitis (VSV), that of hepatitis B (HBV) and C (HCV), as well as parasites such as Trypanosoma cruzi and Plasmodium falciparum. These studies in relation to other biological aspects such as the storage, transfusion and commercialization of blood components become of big importance in the processes of sterilization of plasma and other blood derivates [18, 19]. Nevertheless, the considerations of the toxic effects to other cellular and immunologic systems represent a challenge to investigate about the selectivity and the efficiency factors of these processes. In spite of the implementation of severe measures to prevent the transmission of pathogen agents by way of sanguine transfusion, a high risk still exists due to the residual permanence of these agents. Therefore, it becomes necessary the development of new and better sterilization methods that inactivate a wide range of pathogens. Some of these methods, which promise interesting results, are the applications of direct photochemical or photosensitized sterilization.

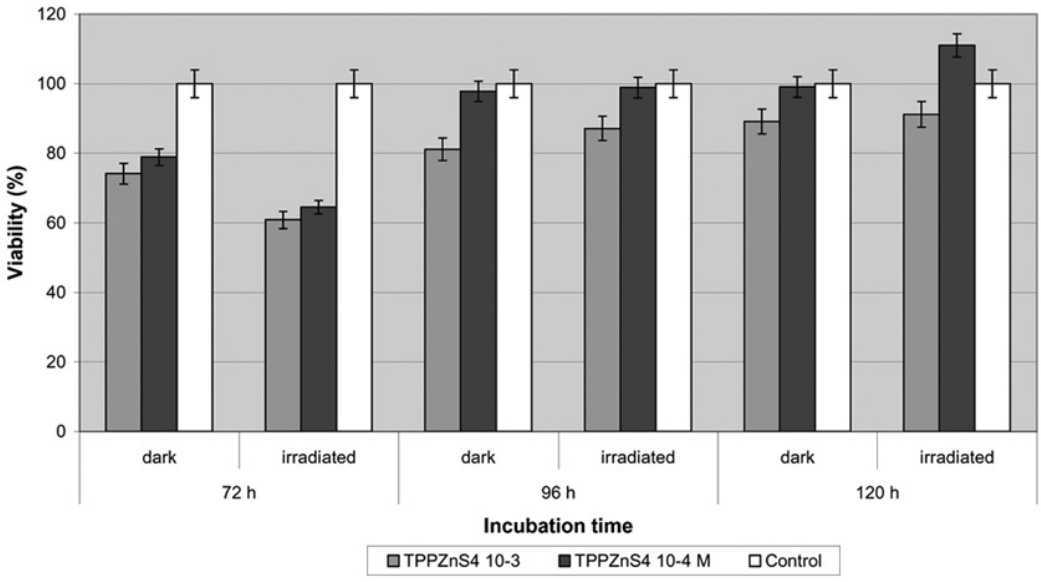

Fig. (9). Effects of $TPPZnS_4$ on the cellular viability under irradiated and dark conditions as determined by a viability assay.

EXPERIMENTAL SECTION

Materials and Methods

All analytical or HPLC grade solvents were purchased from Merck (Darmstadt, Germany). Tetraphenylporphyrin (TPP), TPPCu(II), TPPZn, TPPMn(III)Cl, TPPFe(III)Cl, TPPNi(II), TPPCo(II), benzaldehyde, pyrrole, propionic acid, histidine, rose bengal, luminol, isoluminol, horseradish peroxidase (HRP), 1-(4,5-dimethylthiazol-2yl)-3,5-diphenylformazan (MTT), RPMI-1640 medium, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), hydrogen peroxide, 30 wt. % solution in water and p-nitrosodimethyl aniline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl).

UV-Vis spectrophotometry of the tetraphenylporphyrin (TPP) and metallotetraphenyl sulfonated porphyrin ($TPPMS_4$) solutions was followed using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company-USA) and also Perkin Elmer Lambda-35 UV-Vis spectrophotometer (USA). The fluorescence spectra were registered with a Shimadzu RF 1501 spectrofluorophotometer and Perkin Elmer LS-45.

The structures of the isolated products were elucidated by ^1H NMR and ^{13}C NMR (Brucker Aspect 3000, 300 and 100 MHZ respectively), FT I.R. (Nicolet DX V 5.07) and mass spectra (Varian Saturn 2000) in connection with a Varian chromatograph equipped with a 30-m capillary (CP-Sil, 8CB-MS). All preparative irradiations (1.6×10^{-2} M) were monitored by liquid chromatography (HPLC, Waters Delta Prep 4000) equipped with an analytic and a preparative C18-Bondapak column. The EPR spectra were recorded at 295 ºK on a Bruker EMX (Germany) spectrometer, experimental error ± 0.004.

Synthesis and Characterization of the TPP and Metalized Porphyrins

All the metalized porphyrins were prepared by chemical synthesis. meso-Tetraphenylporphyrin (TPP) was synthesized by means of the following method: benzaldehyde (67 ml) and pyrrole (47 ml) were added simultaneously to refluxing propionic acid (3.0 l) and the mixture was refluxed for 1 hour before being allowed to cool and stand at room temperature overnight. The product was filtered off and washed with water and methanol to give purple crystal (20.5 g, 20%) and compared with the TPP commercially available from Sigma-Aldrich.

The metalloporphyrins (TPPCu, Zn, Pd, Mn, Fe, Ni and Co) was synthesized by means of the following method: To the TPP (300 mg) in boiling $CHCl_3$ (100 ml) was added a saturated solution of the metal-acetate in methanol (1 ml). After a 10 min refluxing and checking by UV-Vis-spectrophotometry, the mixture was concentrated, diluted with a little methanol, and after cooling the metal-complex was filtered off in virtually quantitative yield.

Also, the inclusion of metal in TPP was carried out heating the porphyrin with a metal salt (divalent metal ions) in chloroform, methanol or acetic acid. Absorption, emission, ^1H-, ^{13}C-NMR and EPR were compared with the parent compounds in the literature [11-15]. EPR spectra were registered by means of a Brucker EMX (Germany) at 28 ºC and compared with some reported data [11]. These were compared with the TPPCu(II), TPPZn, TPPMn(III)Cl, TPPFe(III)Cl, TPPNi(II), TPPCo(II) commercially available from Sigma-Aldrich (St. Louis, MO, USA).

General Procedure of the Sulfonation Reaction

In a flask of 5 mL, equipped with magnetic, coolant agitator and a trap for humidity, 10 mg of the metallotetraphenylporphyrin (TPPM) is placed (0.02 mmol) with 1 ml of H_2SO_4 (96%) and allowed to react at 60 ºC during 1 hr. The reaction mixture is allowed to cool down, it is diluted with cold water (50 mL), and neutralized with Na_2CO_3. Extraction with CH_2Cl_2 was necessary to eliminate the TPP in excess [13]. The aqueous phase was evaporated to dryness and methanol was added to dissolve the sulfonated product. The reaction was monitored at different time intervals by TLC and HPLC. The separation of the sulfonated products was made by means of column chromatography (reverse phase, Polygoprep 100-50 and Bondapak C18 column) with elution gradients of H_2O:MeOH. The purification of each one of the compounds required more than one separation in column. The absorption spectra of the **$TPPS_4$** (1.0×10^{-5} M in H_2O, pH 7.2) showed two main bands: 413 nm (1.9) and 515 nm (0.2) with a molar extinction coefficient of $\varepsilon_{max} = 129 \times 10^3$ M^{-1} cm^{-1} ± 10 at λ_{max} = 413 nm. Also 633, 579 and 553 as Q-bands [20-21]. Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 640 nm (947) at λ_{exc} 430 nm [1.00×10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield $\phi_f = 0.120$.

I.R. (KBr): 3360, 3320, 3010, 1940, 1630, 1548, 1390, 1300, 1110, 1060, 995, 739, 700, 638 cm^{-1}. ^1H-NMR (300 MHz, D_2O): δ ppm = 10.00 (m, 6H), 8.20 (m, 8H, H-*m*-phenyl), 8.00 (m, 8H, H- *o*-phenyl), 7.30 (s, 2H, H-pyrrolic), 6.95 (d, 2H, J= 5.50 Hz, H-pyrrolic), 6.60 (d, 2H, J= 5.50 Hz, H-pyrrolic), 6.40 (s, 2H, H-pyrrolic). ^{13}C-NMR (300 MHz, D_2O): δ = 155.50 (2C-pyrrolic), 152.00 (2C-pyrrolic), 143.00 (4C-phenyl), 140.00 (2C-phenyl), 138.00 (2C-phenyl), 137.30 (2C-pyrrolic), 131.33 (8CH-*o*-phenyl), 129 (4CH-pyrrolic), 126.00 (8CH-*m*-phenyl), 118.00 (2C-pyrrolic), 114.20 (4CH-pyrrolic), 81.00 (2C), 74.5 (2C).

Data similar to the 5,10,15,20-tetraphenylporphine-p-tetrasulfonic acid tetrasodium hydrate commercially available from Sigma-Aldrich (St. Louis, MO, USA) and reported data [20-21].

TPPZn(II)S_4

UV-Vis (1.0×10^{-5} M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 421 (1.74, ε = 174000 M^{-1} cm^{-1}). 556 (0.06, ε = 6000 M^{-1} cm^{-1}). Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 605 nm (817) at λ_{exc} 436 nm [1.00×10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield $\phi_f = 0.103$.

I.R. (KBr): 3360, 3300, 3090, 3010, 1936, 1626, 1613 , 1550, 1520, 1389, 1302, 1295, 1155, 1057, 993, 763, 700, 699 cm^{-1}. ^{13}C-NMR (300 MHz, D_2O): δ = 159.30 (2C-pyrrolic), 156.00 (2C-pyrrolic), 151.50 (2C-phenyl), 147.00 (2C-phenyl), 146.00 (2C-phenyl), 142.30 (2C-pyrrolic), 140.00 (2C-phenyl), 138.00 (2C-phenyl), 134 (2CH-pyrrolic), 133.00 (4CH-*m*-phenyl), 131.73 (8CH-*o*-phenyl), 128.00 (2CH-

pyrrolic), 126.00 (2CH-pyrrolic), 125.00 (4CH-*m*-phenyl), 117.00 (2C-pyrrolic), 110.20 (2CH-pyrrolic), 79.00 (2C), 61.00 (2C). EPR: 2.027‹*g*›.

TPPCu(II)S$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 412 (1.12, ε= 112000 M^{-1} cm^{-1}). 539 (0.04, ε = 4000 M^{-1} cm^{-1}). Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 623 nm (702) at λ_{exc} 332 nm [1.00 x 10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield ϕ_f = 0.089. EPR: 2.20 ‹*g*›.

TPPPd(II)S$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 412 (1.27, ε= 127000 M^{-1} cm^{-1}). 520 (0.11, ε = 11000 M^{-1} cm^{-1}). Emission spectra: under the detection limit.

TPPMn(II)S$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 466 (0.31, ε= 31000 M^{-1} cm^{-1}). 562 (0.04, ε = 4000 M^{-1} cm^{-1}). Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 614 nm (705) at λ_{exc} 327 nm [1.00 x 10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield ϕ_f = 0.097. EPR: 2.020 ‹*g*›.

TPPFe(III)ClS$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 394 (0.27, ε= 27000 M^{-1} cm^{-1}). 527 (0.02, ε = 2000 M^{-1} cm^{-1}). Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 619 nm (638) at λ_{exc} 330 nm [1.00 x 10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield ϕ_f = 0.081. EPR: 2.070 ‹*g*›.

TPPNi(II)S$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 413 (0.51, ε= 51000 M^{-1} cm^{-1}). 513 (0.02, ε = 2000 M^{-1} cm^{-1}). Emission spectra: $\lambda_{max\ emiss}$ (intensity) = 641 nm (794) at λ_{exc} 343 nm [1.00 x 10^{-5} M] in H_2O, pH 7.2. The relative fluorescence quantum yield ϕ_f = 0.100.

TPPCo(II)S$_4$

UV-Vis ($1.0x10^{-5}$ M in H_2O, pH 7.2, ε = molar extinction coefficient) showed two main bands: 424 (2.7, ε= 270000 M^{-1} cm^{-1}). 538 (0.13, ε = 13000 M^{-1} cm^{-1}). Emission spectra: under the detection limit. EPR: 2.080 ‹*g*›. Similar spectroscopic data were obtained for the other sulfonated metalloporphyrins and of equal it forms compared with those reported in the literature [11-15, 20-22]. $TPPCuS_4$, $TPPMnS_4$, $TPPFeClS_4$, $TPPNiS_4$, $TPPCoS_4$ are paramagnetic compounds. The EPR spectra of all the metal(II)porphyrins were measured at the X-band frequency to understand both the electronic and bonding feature between the paramagnetic metal and the porphyrinato moiety.

Irradiation

All processes of irradiation were carried out using an illuminator Cole Palmer 41720-series keeping a distance of 10 cm between the lamp surface and the solution flask, varying the time periods of exposure at 37 ºC under continuous shaking., with an emission maximum in UVA-Vis 320-600 nm (3.3 mW/cm^2, 45.575 Lux/seg) (radiation dose 4.5 J/cm^2) as measured with a model of UVX Digital Radiometer after 1 h continued illumination.

Quantum Yields

The relative quantum yields of fluorescence for the TPPMS complexes were determined at room temperature either by comparing the corrected fluorescence intensity of the these complexes in ethanol-H_2O with that of rhodamine B (at a concentration of 1 x 10^{-6} M in ethanol; fluorescence quantum yield, 0.69) or else with that of quinine bisulfate in 0.05 M H_2SO_4 (fluorescence quantum yield, 0.55) [23].

Singlet Oxygen Generation

Indirectly, photosensitized degradation of histidine was measured in the presence of 0.25, 0.50, 1.0, and 1.5 x 10^{-5} M solutions of TPP and $TPPMS_4$ [24]. These solutions were mixed with an equal quantity of L-histidine solution at 0.60 to 0.74 mM in phosphate buffer 0.01 M, pH 7.4. Samples of this mixture were irradiated at time intervals from 60 to 180 min. with the respective controls being protected from light. The concentration of histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The optical density was read on a spectrophotometer at 440 nm against a blank reagent, a modified Pauly reaction and by bleaching of p-nitrosodimethylaniline [25, 26].

Another "trap" method has been successfully used to detect generated $^1O_2$ in a variety of samples. This method is based upon following the consumption of a chemical trap (Furfuryl alcohol, FFA) that react with singlet oxygen. The consumption of FFA was followed by HPLC using a 90:10 H_20/CH_3CN mobile phase composition. The wavelength detection used for monitoring FFA consumption was at 222 nm. Rose bengal, a well known $^1O_2$ sensitizer, was used as a standard for comparison with TPP and TPPSM for $^1O_2$ formation, under identical conditions of photolysis [26, 27].

Generation and Detection of Others Reactive Oxygen Species

Chemiluminescence (CL) was generated in cell-free systems; H_2O_2-induced CL (as a blank): H_2O_2 (3.5 mM) was added to phosphate buffered saline solution (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.2) and luminol (250 μM, prepared daily in 2 M NaOH and diluted with PBS). The $TPPMS_4$-induced CL at different concentrations was dispensed after irradiation in presence of NADPH. The generated CL at 37 ºC was measured continuously for 10 min in a Luminoskan Ascent luminometer (ThermoLabsystems, Finland) in a 96-well Thermo Labsystems Microtiter plate [28-30].

Cells and Virus

The MT4 cells were cultivated in RPMI-1640 medium supplemented with 10% PBS, and penicillin/streptomycin. Cultures were subcultured every 3 days and for the drugs assay the cells were harvested every day two of cultured in order to use the exponential phase of growth. The HIV-1 (HXB2) was obtained through the acquired immune defi-

ciency syndrome-AIDS research and reference reagent program, division of AIDS, National Institute of Allergy and Infectious Diseases-NIAID, National Institutes of Health-NIH. Human T-Lymphotropic Virus Types I & II - HTLV-IIIB/H9 from Dr. Robert Gallo [31].

Citotoxicity Assay

To determine the cellular toxicity (IC50) of the compounds, we evaluate different concentrations of them in a MT4 cell culture. The cells were seeded in 96 well/plate at a density of 30.000 cell/well, the different drugs dilution were added to each well and illuminated with 3.3 mW/cm^2, 45.575 Lux/seg (radiation dose 4.5 J/cm^2) for 30 min, control were established with drugs without illumination and without drugs illuminated. After 72, 96 and 120 h. the cultures were evaluated with MTT cell proliferation assay to establish the fraction of death cells in each concentration [32].

Viral Inhibition

The MT4 cells were seeded in 24 well/plate at a density of 120000 cells/well and the different compounds were added at IC50 level or below, then were infected with HIV-1 IIIB at a mol of 0.1 then they were illuminated or else kept in the dark. 12 h later the supernatant were changed and the cells washed out with PBS tree times and reseeded in a final volume of 3 ml. The virus production was evaluated at 72, 96 and 120 h by collection and replaced of 100 µl of supernatant. The virus determination was performed by p24 detection in a non commercial ELISA. The results were expressed relative to the control [32].

Statistical Treatment of Results

At least three independent experiments were performed except where indicated otherwise. The results are expressed as a mean ± S.E.M. derived from 3-4 observations. The level of significance accepted was $p \leq 0.05$.

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