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Synthesis and pharmacological evaluation of free-base 5,10,15,20-tetrakis (4-hydroxyphenyl)porphyrin and its Zn, Cu, Co metalloderivatives

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In this study, the 5,10,15,20-tetrakis-(4-hydroxyphenyl)porphyrin (TpOHPP) and its Zn, Cu, and Co complexes have been synthesized and evaluated as photosensitizers and for anti-inflammatory activity. TpOHPP and its Zn (Zn-TpOHPP), Cu (Cu-TpOHPP), and Co (Co-TpOHPP) ligands have been synthesized using the Alder-Longo method; their photosensitizer activity has been screened by irradiating HeLa cells with red light, comparing their relative activity to those of protoporphyrin IX (PpIX) and cisplatin (CDDP). The edema mouse model in which inflammation is induced with 12-O-tetradecanoylphorbol 13-acetate (TPA) has been adopted. Finally, molecular modeling (docking) on the test drugs have been conducted using the Autodock VINA software. TpOHPP induced time-dependent cell replication inhibition when the cells are exposed to red light (IC_{50} = 16.35 µM, 2.3 µM, and 2.1 µM at 0, 15, and 30 minutes, respectively). Comparatively, Zn-TpOHPP exerts essentially the same cell replication inhibitory profile with and without red light exposure (IC_{50} = 1.35 µM and 1.38 µM respectively). The percent inhibition of inflammation determined for the test molecules are 54.16% for TpOHPP, 60.38% for Zn-TpOHPP, 67.10% for Cu-TpOHPP, and 67.53% with Co-TpOHPP. TpOHPP and its Zn complex show anti-proliferative activity at low micromolar levels and the four porphyrins exerted significant anti-inflammatory activity *in vivo*.

Keywords: Photodynamic therapy, Photosensitizer, Cancer, Anti-inflammatory, Porphyrin, Metalloporphyrin

Cancer is a disease that is caused by changes in genes, characterized by an abnormal, fast, and uncontrollable proliferation of cells that may or may not spread into surrounding tissues, and develop mechanism to avoid apoptosis (programmed cell death). If left untreated, this process continues until it forms abnormal masses of tissue known as tumors[1]. Currently, cancer occupies a very important place in the statistics of deaths, and several anti-cancer drugs, such as CDDP, are used for its treatment. However, these drugs have some side effects, such as high toxicity and low selectivity toward malignant cells as well as, even currently, patients who exhibit resistance to them[2]. These side effects indicate the necessity to look for new molecules that present fewer side effects and have the same or even better effectiveness as those drugs currently in use.

Cancer is the second leading cause of death globally. Among men, lung, prostate, colorectal, stomach, and liver cancer are the most common types, while breast, colorectal, lung, cervical, and stomach cancer are the most common types in women[3]. Cervical cancer is the second most common cancer among women worldwide[4], and it kills 260,000 women annually[5], with surgery, chemotherapy and radiation as the foremost treatments, although they are also used in combination[6]. Due to the severe side effects and other complications accompanying present-day treatment methodologies, alternative therapies have been developed to minimize or eliminate these problems. Some of these treatments include immunotherapy[7]and photodynamic therapy (PDT)[8]. This therapy has demonstrated therapeutic potential in the treatment of premalignant lesion cervical intraepithelial neoplasia and malignant lesions such as carcinoma *in situ* as well as invasive cervical cancer at early stages[9].

PDT is used to treat both oncological and non-oncological conditions[8]. Some of these oncological conditions are skin cancer, lung cancer, cervical cancer and even cancers more difficult to treat such as glioblastoma[10-13]. PDT is a non-invasive technique with cytotoxic activity (phototoxicity) targeted to malignant cells. The procedure requires the administration of a photosensitizer (PS), followed by

irradiation on the affected zone with light that possesses an adequate wavelength. This approach causes a photochemical reaction that generates oxygen reactive species and subsequently induces cellular death through apoptosis or autophagy. A PS is a chemical compound that is capable of absorbing electromagnetic radiation and initiating a photochemical reaction. Most PSs are tetrapyrrolic macrocycles, similar to those found in nature as a heme group, which is the prosthetic group of hemoglobin. The first PS utilized clinically was porfimer sodium, commercially known as Photofrin[14]. This compound is a derivative of hematoporphyrin and represents the first generation of PSs. Porfimer sodium is a mixture of monomers, dimers, and oligomers, and it is available worldwide. This PS has been the most used and therefore possesses more clinical history than any other. However, it has some serious disadvantages (*e.g*., slow elimination as well as its complex composition through which some of the monomers, dimers, and oligomers previously mentioned do not exert any biological activity) that have caused the development of second and third generation PSs[15].

Due to its photophysical properties and major affinity for malignant cells, porphyrins have been widely used in many studies as PS agents in PDT[16,17]. Some 5,10,15,20-tetraphenylporphyrin-derivatives have shown good phototoxic activity that is showed when porphyrin is irradiated with red light, particularly those having hydroxyl substituents in phenyl groups[18,19]. There are also numerous examples of metal-based PSs that have demonstrated interesting bioactivity *in vivo* and *in vitro*. In fact, some of these PSs are currently in clinical trials, and others, such as Purlytin, are being used in PDT[20].

Currently, there are studies that show a strong relationship between the use of non-steroidal anti-inflammatory drugs (NSADs) and a decrease in the incidence of cancer. These studies are focused on colorectal and breast cancer; however, there is strong evidence that other types of cancer can be prevented by the consumption of NSADs, which is why it is interesting to test compounds with anti-inflammatory activity in the treatment of cancer[21].

Both 5,10,15,20-tetraphenylporphyrin-derivatives and their complexes, formed with divalent metallic cations, can be easily synthesized[22-24], making it possible to obtain and to probe the biological activities of a variety of compounds. The use of essential metals instead of non-essential ones to form the respective complexes may be a good option due to the structural resemblance to endogenous human body metabolites required for different biological functions[25,26], probably resulting in a lower toxicity and an easier biotransformation and elimination from the body.

In this work, we present the synthesis, structural characterization, and biological activities of both anti-inflammatory and PS agents of TpOHPP and of its Zn, Cu, and Co metalloderivatives.

Results and Discussion

Synthesis and spectroscopy of free-base porphyrin and its Zn, Cu, and Co complexes

The synthetic route followed to synthesize TpOHPP, Co-TpOHPP, Cu-TpOHPP y Zn-TpOHPP is shown in Fig. 1. Compound TpOHPP was obtained by the Adler-Longo method[27], *via* aromatic electrophilic substitution condensing pyrrole and an aromatic aldehyde in propionic acid at boiling temperature in the presence of atmospheric oxygen. Metalloderivatives were obtained stirring TpOHPP with metallic acetate in methanol at boiling temperature. This procedure allowed the insertion of Zn, Cu, and Co into the porphyrin ring.

The UV-visible spectrum of TpOHPP was of Etio type, showing four Q bands, and the band at 650 nm was responsible for the absorption of the red light. Nevertheless, as it has been previously reported, the four Q bands disappeared for the metalloderivatives and two new bands, called β and α bands, appeared[28]. These bands were below 600 nm. This result might be due to the attraction of electronic density by a metallic cation, resulting in changes in the resonance pathway.

Molecular docking

Due to the important attention that the heme oxygenase-1 has received in recent years as an emerging target for cancer therapy[29] coupled with a previous work reported by Nowis *et al*. that shows zinc protoporphyrin IX (PpIX) exerts potent antitumor effects by acting as a selective heme oxygenase-1 inhibitor[30], we decided to carry out an *in silico* study in order to determine the possible docking sites of Zn-TpOHPP.

In Fig. 2 we can see that Zn-TpOHPP binds to the active site of the protein with an affinity of −7.5 kcal/mol. It is stabilized by several important

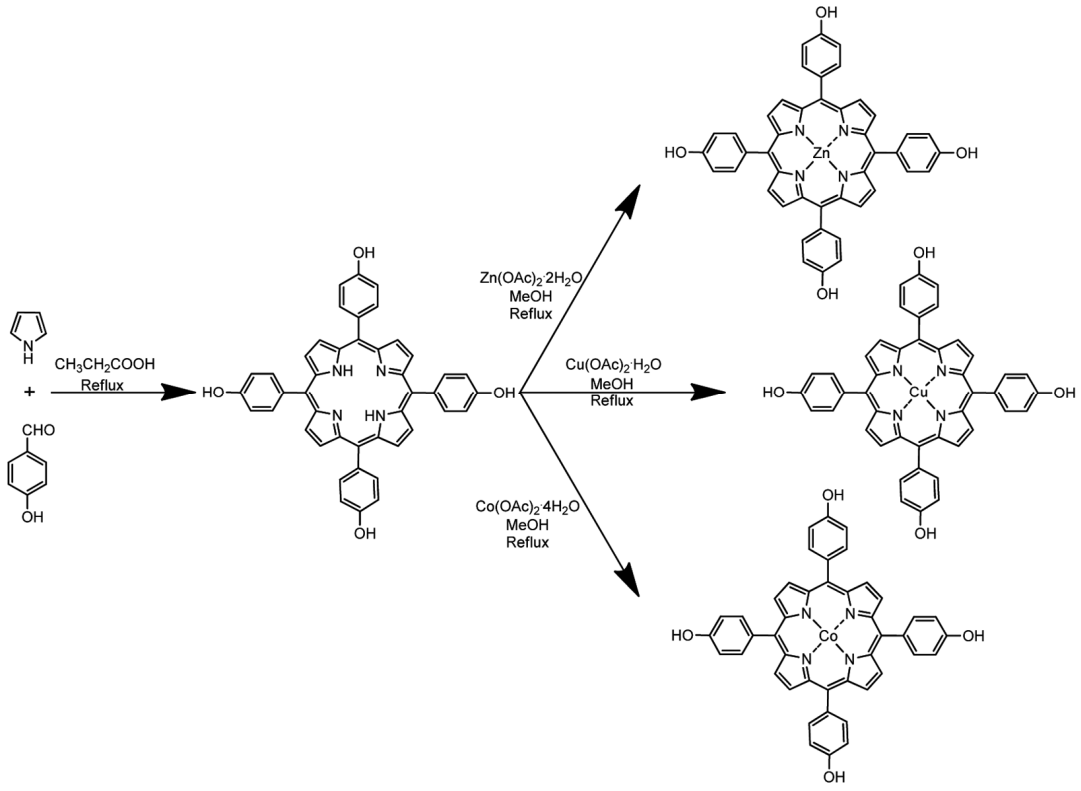

Fig. 1 — The synthesis of TpOHPP, Co-TpOHPP, Cu-TpOHPP, and Zn-TpOHPP

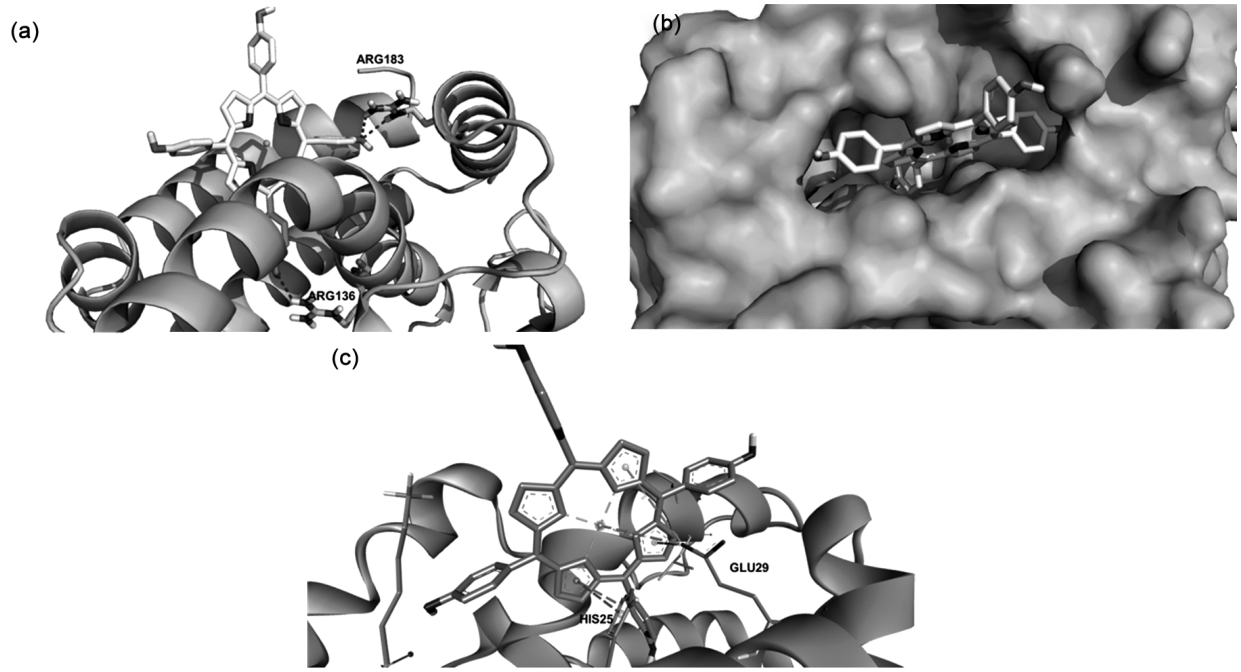

Fig. 2 — Binding mode of Zn-TpOHPP with the active site of heme oxygenase-1. Images were generated using PyMOL 1.3 and Discovery Studio v16.1.0.15350. **A**. Hydroxyl groups of porphyrin form hydrogen bonds with residues Arg136 and Arg183. **B**. Porphyrin seen from outside of the active site. **C**. The pyrrole ring presents π-π interactions with the residue His25. Similarly, we can observe π-cation interactions with the Glu29 residue and a metal-cation interaction between Zn and Glu29

ligand-receptor interactions within the pocket of the active site. For instance, three hydrogen bonds are formed between two OH groups, present in the ligand, with the guanidine group of the residues Arg136 and Arg183 (A). Also, it can be observed the formation of π-π interactions of the pyrrole ring with the imidazole ring present in the residue His25. Other kind of interactions occurred between the pyrrole ring and the carboxylate group of Glu29, wherein this is a π-cation type interaction. Finally, another interaction is metal-cation type, which is present between the Zn atom and the carboxylate group of Glu29 (B). All these interactions allow us to suppose that the ligand-protein stability is very high.

Phototoxicity and intrinsic cytotoxicity of the synthesized compounds

The light system did not exert toxicity at any of the radiation times (0-30 min). Compounds Cu-TpOHPP and Co-TpOHPP did not show both cytotoxicity, that is showed when the compound is tested without irradiation of red light, and phototoxicity at the concentrations used in these tests (IC_{50} 0-32 μM). Otherwise, TpOHPP showed different IC_{50} values depending on the time exposed to radiation (Table 1).

The porphyrins TpOHPP and Zn-TpOHPP exerted notable anti-proliferative effects on HeLa cells. The phototoxicity of TpOHPP increased with the time of exposure of the cells to red light (Table 1). The IC_{50} value was 2.1 μM at 30 minutes of radiation, which was smaller than that obtained at 15 min (2.3 μM). The activity of this compound was greater than that obtained with PpIX (IC_{50} 5.37 μM and 3.98 μM at 15.0 and 30 min, respectively).

Both IC_{50} values at 15 and 30 minutes showed statistically significant differences when compared to the IC_{50} (16.35 μM) obtained in the absence of red light (t-Student + Tukey, $P< 0.05$). This result means that the phototoxic effect is strongly influenced by the time of exposure of the cells previously treated with this porphyrin.

However, the porphyrin Zn-TpOHPP had intrinsic cytotoxicity, but it did not exert phototoxic activity at 15 minutes of radiation. The phototoxic effect occurred until 30 min of exposure to red light was reached, although the bioactivity only increased slightly (Table 1). This result could be due to the disappearance of the Q band of interest (650 nm), which is present in the UV-visible spectrum of its free base and in the appearance of α band (600 nm). This fact suggests that the metallic derivative had poor ability to absorb red light, considering that the wavelength used was 650 nm. Despite, the results indicate that this compound might be used in later studies without irradiation. The cytotoxicity of Zn-TpOHPP (IC_{50} 1.38 μM) was better than that obtained with CDDP (IC_{50} = 3.5μM). These results suggest that these two porphyrins have different mechanisms of action.

As it was mentioned previously, the biological activity of Zn-TpOHPP is explained by the action of it as a competitive inhibitor of heme oxygenase-1. In addition to the π-π and π-cation interactions that contribute to the stabilization of the ligand-protein complex, the nature of the binding metal seems to determine whether the porphyrin ring will bind or not to this enzyme through a metal-cation interaction; in this case, only Zn-TpOHPP was able to do so. As a result, this might induce the inactivation of the heme oxygenase-1 by competitive inhibition with the heme group, inducing a significant accumulation of reactive oxygen species and the subsequent death of cells. It is well known that this enzyme is involved in the degradation of heme group and that its products can present cytoprotective effects. Furthermore, this enzyme is present in several tumors at raised quantities.

In general, both porphyrins presented lower IC_{50} values when compared with the IC_{50} obtained from both PpIX and CDDP, which were used as positive controls (Table 1). As CDDP, in combination with 5-fluorouracil, is often thought to be the gold standard of treatment for locally advanced cervical cancer[31], these findings deserve to be taken into consideration in further studies.

Table 1 — IC_{50} values of compounds at different times of radiation and the dark toxicity. For CDDP, the symbol "/" indicates that the incidence of red light did not have any influence on bioactivity. For the rest of compounds indicates that they did not exert any bioactivity at the different times of radiation

Compound	IC_{50} (μM)		
	Dark toxicity	Red light (15 min)	Red light (30 min)
PpIX	33.11	5.37	3.98
CDDP	3.50	/	/
TpOHPP	16.35	2.3	2.1
Zn-TpOHPP	1.38	1.35	1.1
Cu-TpOHPP	/	/	/
Co-TpOHPP	/	/	/

Anti-inflammatory effects

***In vivo* model acute TPA-induced ear edema**

Indomethacin significantly decreased the size of ear edema induced with TPA in mice by 65.43% in

comparison with the vehicle group, while Cu-TpOHPP, Co-TpOHPP, Zn-TpOHPP,and TpOHPP also significant decreased the edema by 67.10%, 67.53%, 60.38% and 54.16%, respectively. There were no statistically significant differences between the positive control and porphyrin groups (Table 2).

These results suggest that the four porphyrins exert the same effectiveness as the indomethacin, decreasing the inflammatory process. According to research from Jelić and coworkers, porphyrins can directly inhibit a non-receptor Src-family tyrosine kinase named Fyn, provoking an anti-inflammatory effect by the inhibition of tumor necrosis factor alpha (TNF-α) production[32]. This outcome provides the opportunity for developing new anti-inflammatory drugs with fewer side effects and with even better potency.

In vitro anti-inflammatory effect

TpOHPP and Co-TpOHPP at concentrations of 3.125, 6.25, and 12.5 µg/mL did not affect cell viability of macrophages (Fig. 3 and Fig. 4); however, at concentrations of 25, 50, 100 and 200 µg/mL of TpOHPP and Co-TpOHPP the cell viability lower at 80% in both treatments. Therefore, the concentration of TpOHPP and Co-TpOHPP used in further experiments was 12.5 µg/mL.

When cells were exposed to 5 µg/mL LPS for 24 h, the NO concentration increased markedly (Fig. 5). TpOHPP and Co-TpOHPP at 12.5 µg/mL inhibited the production of NO by 40.7% and 35.2%, respectively. This effect of TpOHPP was similar to that obtained with IND at the same concentrations (44.8% and 40.7%, respectively).

Overproduction of NO during inflammation can activate nuclear factor‑kappa B and induce the expression of proinflammatory mediators. The increased levels of NO by inducible NO synthase can result in tissue damage[33]. Thus, inhibition of NO production in macrophages is a therapeutic strategy for inflammation. In this study, TpOHPP

Table 2 — Anti-inflammatory activity of TpOHPP, Co-TpOHPP, Cu-TpOHPP, and Zn-TpOHPP in ear edema-induced with TPA in mice

Treatment	Dose (mg/ear)	Inhibition percentage (%)
Vehicle	–	0
Indomethacin	2	65.43 ± 2.98*
TpOHPP	2	54.16 ± 3.78*
Co-TpOHPP	2	67.53 ± 5.27*
Cu-TpOHPP	2	67.10 ± 3.38*
Zn-TpOHPP	2	60.38 ± 3.92*

The values expressed as Mean ± S.E.M. (n=8). *p<0.05 *vs* vehicle.

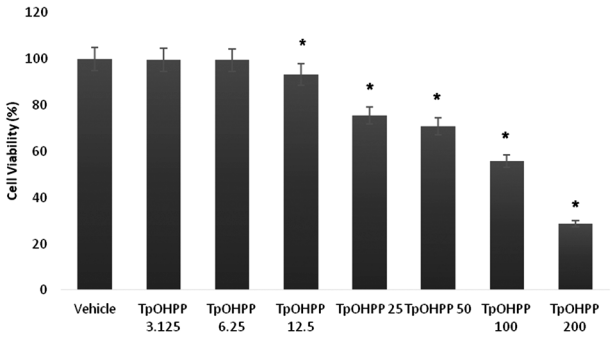

Fig. 3 — Effect on the cell viability of macrophages treated with TpOHPP at 3.125, 6.25, 12.5, 25, 50, 100 and 200 µM/mL. Results are expressed as the percentage of surviving cell relative to control cell. The results are the mean of three determinations ± S.E.M. * p <0.05 *vs* vehicle

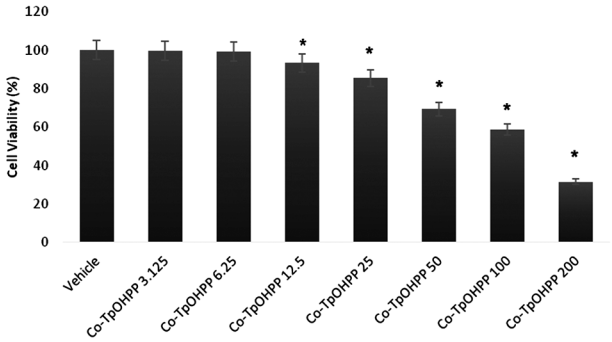

Fig. 4 — Effect on the cell viability of macrophages treated with Co-TpOHPP at 3.125, 6.25, 12.5, 25, 50, 100 and 200 µM/mL. Results are expressed as the percentage of surviving cell relative to control cell. The results are the mean of three determinations ± S.E.M. * p <0.05 *vs* vehicle

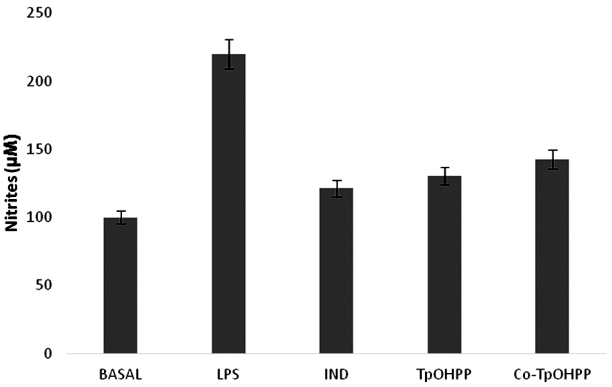

Fig. 5 — Effects of TpOHPP and Co-TpOHPP at concentration of 12.5 µM/mL on the production of nitric oxide in lipopolysaccharide‑stimulated macrophages. The values are the mean ± standard error of mean of three independent experiments. * P < 0.05 *versus* vehicle

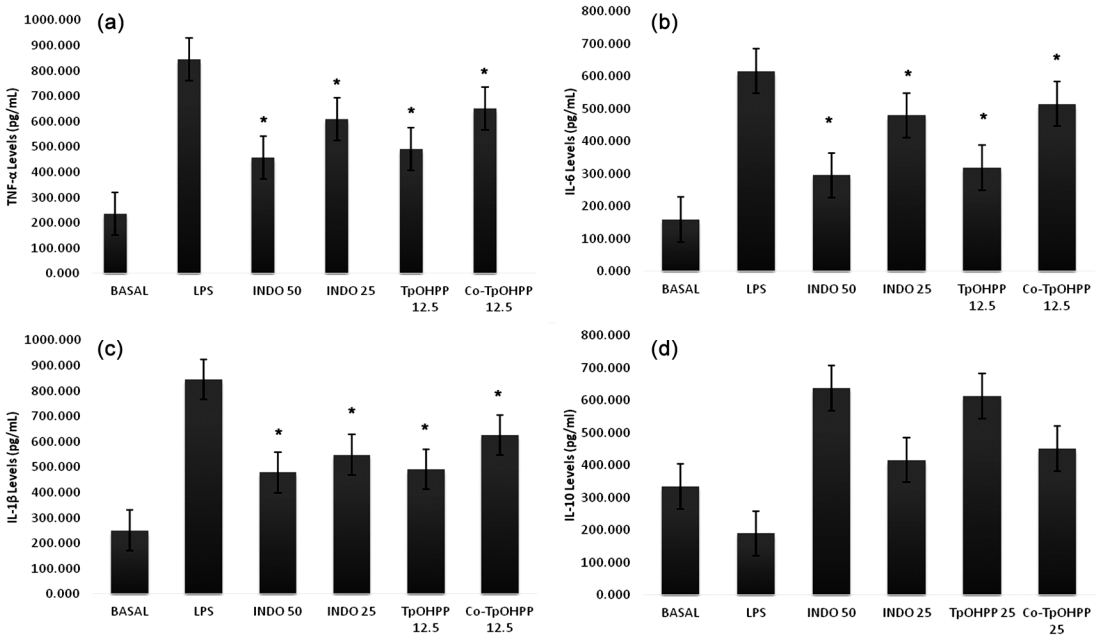

Fig. 6 — Effects of TpOHPP and Co-TpOHPP on the levels of a) TNF-α, b) IL-1β, c) IL-6 and d) IL-10 the results are mean of 3 determinations ± S.M.E. *$p<0.05$ *vs* vehicle

significantly inhibited NO production in LPS‑stimulated macrophages.

The effects of TpOHPP, Co-TpOHPP, and indomethacin on the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were measured in a culture medium of macrophages stimulated with LPS (Fig. 6). In this study was found that TpOHPP at concentration of 12.5 µg/mL significantly reduced the concentrations of TNF-α (1.7-fold), IL-1β (1.7-fold) and IL-6 (1.9-fold), the effects were similar to those obtained with indomethacin. In the case of Co-TpOHPP at concentration of 25 µg/mL (Fig. 6) reduced the concentrations of TNF-α (1.2-fold), IL-1β (1.1-fold) and IL-6 (1.3-fold), the effects were lower to those obtained with the reference drug.

Lipopolysaccharide (LPS) interact with the membrane receptor CD14 to induce the production of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6[34]. TNF- α is a trimeric protein encoded within the major histocompatibility complex, prevent infections and keep inflammation localized, an excessive production of this mediator can be dangerous[35]. IL-6 is a mediator produced in response to infections and tissue injuries, help host defence through the stimulation of acute phase responses, hematopoiesis, and immune reactions[36]. IL-1β is a pro-inflammatory cytokine that has been implicated in pain, inflammation, and autoimmune conditions and is responsible of increased damage during tissue injury[37]. The decrease in the levels of these type of cytokines is due to the inhibition of NF-κB (nuclear factor kappa B)[38]. This fact suggests these porphyrins can act on the transcription factor, but studies are required for these compounds to act in this pathway.

There are reports in the literature that non-steroidal anti-inflammatory drugs are effective to some extent in cancer because they avoid the over expression of COX-2, which is known to promote angiogenesis in tumors when it is over-expressed[39], or it can increase the resistance of cells to apoptosis[40]. This outcome indicates that a compound with anti-inflammatory activity may be a candidate in the treatment of cancer.

Toxic effects

Mice administered with a dose of 2500 showed a decrease in appetite and thirst. The dose of 1250 mg/kg or lower might be considered as safe due to the absence of toxic effects. The animals were observed during 72 h and, after this period, the animals were sacrificed and macroscopic analysis of the internal organs showed no damage. These results might indicate these compounds would be better tolerated by the digestive system due to the absence of gastric ulcers in mice, which are usually caused by

indomethacin. However, chronic toxicity studies should be performed in order to test the safety of these molecules for long-term administration.

Experimental Section

Chemicals

Pyrrole and 4-hydroxybenzaldehyde were purchased from Sigma-Aldrich. Zinc acetate dihydrate was obtained from Mallinckrodt. Cooper acetate monohydrate and Cobalt acetate tetrahydrate were obtained from J.T. Baker. CDDP was obtained from TEVA and Protoporphyrin IX from Toronto Research Chemicals. DMEM was purchased from Caisson Laboratories. Fetal bovine serum was obtained from Corning. Trypsin-EDTA, penicillin-streptomycin, TPA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich.

Structural characterization

^{1}H NMR data was obtained with an Agilent DD2 600 spectrometer, using DMSO-d_6 as solvent and TMS as reference. UV-Vis data was obtained with aShimadzu UV-1800 spectrophotometer, using methanol as solvent.

5,10,15,20-Tetrakis(4-hydroxyphenyl)porphyrin

It was obtained as dark blue crystals (Yield 23.5%). m.p.>300°C. UV-Vis (MeOH) λ_{max}(log ε) 416, 554, 592, 650 nm; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.96 (4H, s, OH), 8.87 (8H, s, H-pyrrole), 8.01-8.00 (8H, d,J = 8.01 Hz, H_{Ar}), 7.22-7.21 (8H, d,J = 8.01 Hz, H_{Ar}), -2.87 (2H, s, NH-pyrrole); LC-MS/MS:*m/z* 678.75 $[M1]^+$.

5,10,15,20-Tetrakis-(4-hydroxyphenyl)porphyrin-Zn(II)

It was obtained as metallic blue bright crystals (Yield 82.5%). m.p.>300°C. UV-Vis (MeOH) λ_{max}(log ε)559, 600 nm; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.80 (4H, s, OH), 8.87 (8H, s, H-pyrrole), 7.93-7.92 (8H, d,J = 7.92 Hz, H_{Ar}), 7.15-7.14 (8H, d,J = 7.92 Hz, H_{Ar}); LC-MS/MS:*m/z* 742.11 $[M1]^+$.

5,10,15,20-Tetrakis-(4-hydroxyphenyl)porphyrin-Cu(II)

It was obtained as metallic red bright crystals (Yield 85.3%). m.p.>300°C. UV-Vis (MeOH) λ_{max}(log ε) 539 and 579 nm; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.80 (4H, s, OH), 8.87 (8H, s, H-pyrrole), 7.93-7.92 (8H, d,J = 7.92 Hz, H_{Ar}), 7.15-7.14 (8H, d,J = 7.92 Hz, H_{Ar}); LC-MS/MS:*m/z* 740.28 $[M1]^+$.

5,10,15,20-Tetrakis-(4-hydroxyphenyl)porphyrin-Co(II)

It was obtained as metallic brown crystals (Yield 80.2%). m.p.>300°C. UV-Vis (MeOH) λ_{max}(log ε) 545 and 584 nm; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.07 (4H, s, OH), 9.01 (8H, s, H-pyrrole), 7.92-7.91(8H, d,J = 7.07 Hz, H_{Ar}), 7.17-7.16 (8H, d,J = 7.07 Hz, H_{Ar}); LC-MS/MS:*m/z* 735.67 $[M1]^+$.

Synthesis of TpOHPP

This compound was synthesized using the Adler-Longo method (Fig. 1)[41]. In a round bottom flask, 140 mL of propionic acid were added and heated to boiling temperature. Then, 0.028 mol of 4-hydroxybenzaldehyde were added to finally, 0.028 mol of pyrrole were added drop-wise, and the reaction mixture was refluxed for 3.5 h. After the reaction was stopped, the reaction crude was left in refrigeration overnight to allow the precipitation of porphyrin. The precipitate was filtered and purified using a Soxhlet equipment, with ethyl acetate as a solvent. Finally, a chromatographic open column was prepared with 50 g of silica gel using as mobile phase a mixture of Hex:AcOEt and the polarity was up on intervals of 5 mL of AcOEt. Finally, the solvent of fraction 30:70 (Hex:AcOET) that contained the porphyrin was removed under vacuum and the solid was recollected and stored in dark.

Synthesis of metalloderivatives

1.46×10^{-4} mol of TpOHPP and 1.46×10^{-3} mol of metallic acetate (Co, Cu and Zn) were added to 80 mL of methanol, and the reaction mixture was refluxed for 5 h. Once the reaction was stopped, the solvent was removed under vacuum, and the respective metalloderivative was isolated with column chromatography performed on silica gel (Hexane:Ethyl acetate). Finally, the solid obtained was washed three times with chloroform.

Molecular docking

Docking studies were performed in order to propose the possible binding mode of Zn-TpOHPP to the heme oxygenase-1. The X-ray crystal structure of the human heme oxygenase-1 (PDB ID: 1n45) was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb).

Chain A was retained, while the chain B, the heme group, and all the water molecules were removed.

All the polar hydrogens and partial charges (Gasteiger charges) were added. The structure was submitted to a geometry optimization, the AMBERff99SB force field was utilized with Chimera v.1.9 software. The ligand was constructed and submitted to a geometry optimization using AM1 force field. To be sure that geometry optimization did not affect the active site of heme oxygenase-1, RMSD (0.105 Å) was calculated by aligning the optimized enzyme against the non-optimized one employing PyMOL (version 1.3).

The calculations were carried out employing Autodock VINA. The selected conformations were those inserted in the active site, most repeated, and with lowest free energies.

Construction of the light system

For the construction of the light system, a strip of 300 red LED lights was used. The strip was cut into pieces of 20 cm and was adhered on a wood table with dimensions of 12×20 cm. Each piece was connected in a series connection. The bases of the table were collocated, leaving 10 cm between the LED lights and the 96-well plate. The irradiance generated by this device was 3.75 J/m^2.

Intrinsic cytotoxicity and phototoxicity of *in vitro* studies

To evaluate the biological activities of these compounds as PSs on HeLa cells, the methodology reported by Hopkins *et al*. was followed[42]. The cells were grown using DMEM supplemented with 10% fetal bovine serum and 100 µL of penicillin and streptomycin (37°C, 5% CO_2), washed with PBS every third day, detached with 10% trypsin-EDTA solution and seeded in a 96-well plate. A Neubauer chamber was used for cell counting. For each experiment, the cells were seeded at a concentration of 5000 cells per well and were kept in incubation overnight to allow the attachment of the cells. All the porphyrins were dissolved in DMSO and diluted with DMEM, and successive dilutions were performed to achieve the desire concentrations. PpIX and CDDP were used as positive controls. The concentration of DMSO per well was 0.1%. After 24 h of incubation, the medium of each well was eliminated, washed with 100 µL PBS and finally replaced with 100 µL DMEM. The cells were irradiated for 15 and 30 min at 37°C and were incubated for 24 h. The sensitivity of the cells to the porphyrins was measured using the MTT assay. First, 10 µL MTT (5 mg/mL) were added per well and incubated for 4 h at 37°C. After this time the supernatant was eliminated, then the formazan crystals were dissolved in 100 µL DMSO. Optical densities were measured at 540 nm using an ELISA plate reader from BioRad. The same methodology was used to determine possible toxic effects by the light system, but the addition of the previously mentioned compounds to establish the intrinsic cytotoxicity cells irradiation was omitted.

Experimental animals

Male CD1 mice (20-25 g, 7-8 weeks of age) obtained from the Universidad Autónoma Metropolitana-Xochimilco animal facility were kept in isolated cages; mice were maintained with food (Lab Diet 5001) and water ad libitum. They were housed in groups at 24°C ±1°C under 12 h light-dark cycles. All experimental protocols were approved by the Research Bioethics Committee of the Universidad Autónoma Metropolitana-Xochimilco with number 140. All animals were cared for and treated humanely according with the current procedure for the care of animals by the official Mexican Norm (NOM-062-ZOO-1999). The animals were acclimatized to laboratory conditions for one week prior to the experiments and they were done after 9:00 am, the number from the Ethical Committee is 177. They were sacrificed in a CO_2 chamber.

Anti-inflammatory effect. *In vivo* model: TPA-induced mouse ear edema

The procedure for TPA-induced mouse ear edema has been described previously[43]. First, 2.5 µg of TPA were dissolved in 25 µL of acetone and topically applied to the inner and outer surfaces of the right ears of the mice, and acetone (25 µL) was applied to both surfaces of the left ear. Thirty minutes later, each porphyrin (2.0 mg/ear) or indomethacin dissolved in acetone was topically applied to the right ear. The animals were sacrificed after six hours, and 6 mm plugs of the central portion of both ears were weighed. The percent inhibition of the edema was determined using the following formula.

$$\% \text{ inhibition} = \frac{(W - Wo)}{(W' - W'o)} \times 100$$

where W= TPA + treatment; Wo= vehicle-treated ear; W'= right ear with TPA, without treatment; W'o= left ear with vehicle, without treatment.

Acute toxicity

The methodology of Lorke was used to determine the acute toxicity[44]. Formulations of porphyrins were orally administered to the groups of mice (n=5) at doses of 2500 mg/kg and 1250 mg/kg. After administration, the rodents were observed under open-field conditions for 72 h. Any animal death or signal of toxicity was registered.

***In vitro* model: determination of pro-inflammatory interleukin levels**

The serum levels of TNF-α, IL-6, IL-10, and IL-1β were measured using a commercially available ELISA kit according to the manufacturer's instructions. The OD was measured using a microplate reader at 405 nm with a wavelength correction set to 650 nm.

Macrophages J774A.1 were grown at a density of 1×10^6 and treated with TpOHPP and Co-TpOHPP at 12.5 μM. Another group was treated with INDO at a concentration of 8.5 and 17 μg/mL (25, 50 μM) and incubated for 2 h. After LPS (5 μg/mL) was added and the cells were incubated for an additional 24 h, the cell-free supernatants were collected and stored at −80°C until they were analyzed by immunoassay to quantify the cytokines. The concentrations of IL-6, IL-1β, and TNF-α in the supernatants of the cultures of J774A.1 cells were determined using a commercial immunoenzymatic kit (PeproTech).

Determination of nitric oxide production

The supernatants collected in the assay of anti-inflammatory activity were used for the determination of nitric oxide. For this assay the supernatant was collected and 100 μL were treated with 100 μL of Griess reagent (1% sulphanilamide, 0.1% naphtylethylenediamine dihydrochloride, and 5% orthophosphoric acid), the mixture was incubated at 37°C for 15 min. Then the absorbance was measured at 540 nm in a microplate reader.

Statistical analysis

Data are expressed as the mean ± S.E.M. Statistical analysis was performed using Student's t-test or One-Way ANOVA, followed by post hoc Tukey test. $P \leq 0.05$ was used to determine significance.

Conclusion

TpOHPP and Zn-TpOHPP present phototoxicity and cytotoxic activity, respectively; however the mechanism by which they act is different.The zinc complex exerted notable intrinsic cytotoxic activity that was even higher than its free base at different times of radiation. Additionally, the four porphyrins in the study presented anti-inflammatory activity in an acute model with a similar effect to the reference drug.

These results open up the possibility of carrying out more studies in a future that help todeterminate the possible action mechanisms that could explain the different behaviors of TpOHPP and Zn-TpOHPP in the presence of light or without it.

On the other hand, it is important stand out the anti-inflammatory activity presented by these four test compounds, so it will be interesting to carry out the determination of this effect in other models and in different doses.

Finally, it is important to evaluate the levels of different anti-inflammatory mediators, to associate these results with a mechanism of action

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Conflicts of Interest

The authors declare no conflict of interest.

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