EVALUATION OF PHOTODYNAMIC ACTIVITY OF METALLOPORPHYRINS ON HUMAN NEUROBLASTOMA CELL LINE

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Summary

Tetraphenyl and tetranitrophenyl base porphyrins were synthesized using microwave irradiation technique and their metallic complexes of copper, zinc, tin and lead were synthesized by conventional methods. The lipophilicity of the well-characterized compounds was evaluated at 25 °C and in vitro photodynamic activity was evaluated with human neuroblastoma cell lines. The photosensitizing efficiency of the metalloporphyrins in photodynamic activity increases in the order of Cu>Pb>Zn>Sn. Among the well-characterized porphyrins and its their metallic derivatives, copper and lead complexes was were found to show better photodynamic activity than zinc and tin porphyrins and this methodology can be used to demonstrate the photodynamic therapy (PDT) procedure in a laboratory experiment.

Key Words: Photodynamic therapy, metalloporphyrins, PDT, photochemical reaction, photoactivation, tumor, neuroblastoma.

Short title: Photodynamic activity of metalloporphyrins on human neuroblastoma cell line.

Introduction

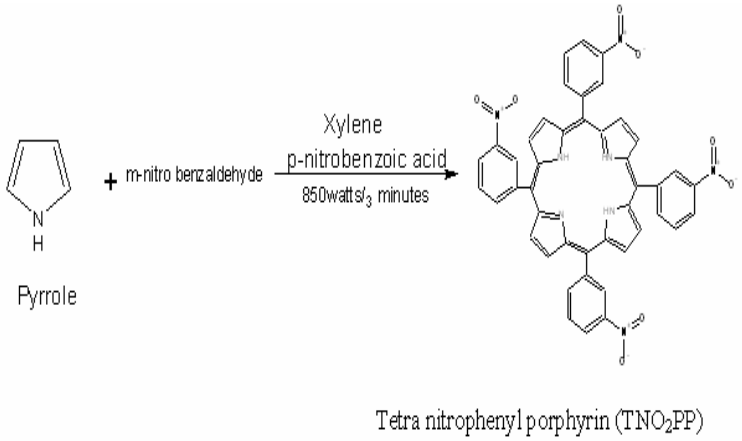
Photodynamic therapy (PDT) is a revolutionary treatment aimed at detecting and treating of various organelle cancers including retinal, brain, kidney, etc., by using certain photosensitizers in conjugation with visible light. Detection and cure of tumors is one of the most recent and promising applications of porphyrins in medicine [1, 2, 3]. After administration of a photosensitizer, which gets selectively retained by tumor cells, the subsequent irradiation with visible light in the presence of oxygen specifically inactivates neoplastic cells. Basically, two types of reactions can occur after photoactivation of the photosensitizer. One involves the generation of free radicals (type I photochemical reaction) and the other involves the production of singlet molecular oxygen, O_2 ($^1\Delta_g$), (type II photochemical reaction). This is the main species responsible for cell inactivation. Evidences favors favor the role of the type II process in cells. Although the photodynamic process of the sensitizers on neoplastic tissues is still not well understood, adequate photosensitizers are deemed to have possessed specific chemical and biological properties. Two important of the photochemical prerequisites for photochemical activity are a high absorption coefficient in the visible region of the spectrum and a long lifetime of triplet excited state to produce efficiently O_2 ($^1\Delta_g$). On the other hand, the metallic derivatives as well as substitutions in the structure of sensitizer, result in an intramolecular polarity axis. This property, which is an important precondition for an effective photosensitization could produce a better accumulation in subcellular compartments [4, 5, 6].

Material and Methods

Experimental Methodology

Synthesis

Tetra-phenyl porphyrin (TPP) and Tetra-nitro-phenyl porphyrin (TNO_2PP) were synthesized by fusing pyrrole with the corresponding aldehyde using microwave irradiation technique [7].



A solution of pyrrole (1.4 ml) and aldehyde (2.1 ml) benzaldehyde / nitrobenzaldehyde) was added in xylene (45 ml) catalyzed by 4-nitro-benzoic acid. The mixture introduced into microwave oven Samsung CE2877N, and heated for 3 minutes in 850 watts setting. After cooling to room temperature, the crude product was recrystallised with ethanol 2-3 times to yield porphyrin.

The metal complexes of Copper, Zinc, Tin and Lead with (TPP) and (TNO_2PP) were synthesized conveniently by different conventional methods [6, 8, 9]. The porphyrinato-complexes of Copper, Zinc and Lead were synthesized by using the same procedure.

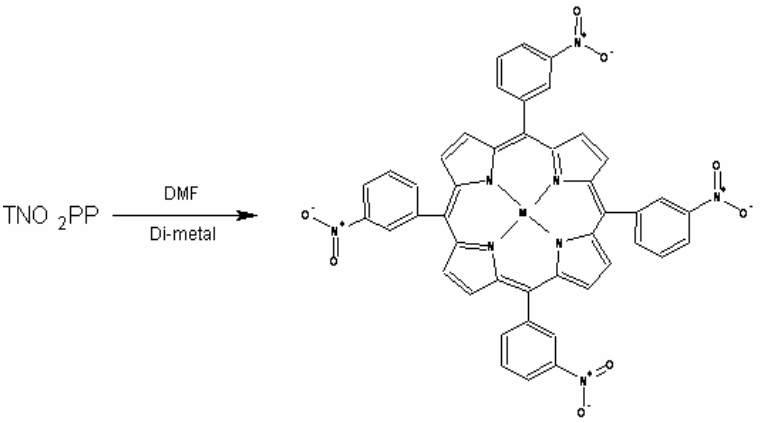
Procedure for [5, 10, 15, 20 tetraphenyl and m-tetranitrophenyl porphyrinato Cu, Zn, Pb] complexes

About 10 ml of Dimethyl formamide (DMF) was taken in an Erlenmeyer flask containing few boiling stones, and it was heated on the hotplate until the solvent DMF boils gently. 100 mg of porphyrin synthesized above was added and allowed it to dissolve completely. To this 40 mg of

Copper (II) acetate, Zinc (II) acetate, Lead (II) acetate was added to the corresponding flask and continued boiling for 10 minutes. Flask was cooled in an ice bath for 20 minutes, and then diluted with 10 ml of distilled water. The solid product was collected by suction filtration, washed thrice with distilled water, and dried again by suction. The product was purified by column chromatography on alumina employing with chlorinated solvent chloroform as the eluent.

Procedure for [5, 10, 15, 20 tetraphenyl and m-tetranitrophenyl porphyrinato Sn] complex

Free-base porphyrin (1.633 mmol) and tin dichloride (4.9 mmol) were dissolved in 200 ml of THF in the presence of 10 gm of molecular sieves (size 4 A°, dried during 2 hours at 250°C under vacuum). Pyridine (5 ml) was added by using a syringe and the reaction mixture was then refluxed for four to six hours with occasional stirring .The metallation reaction was monitored by UV-Visible spectroscopy simultaneously and after completion of the reaction, the bluish green solution was filtered out and then evaporated under reduced pressure until the crude product was obtained. The raw material was redissolved in toluene and was purified by column chromatography. It was done over an alumina-packed column, which was previously dried for 2 hour at 250°C under vacuum.



Metal complex of tetra nitro phenyl Porphyrin
Where M =Cu, Zn, Sn or Pb

Purification

The crude product of metal-free base as well as metallic porphyrins was purified by column chromatography dissolved with minimum amount of chloroform and chromatographed on a 60x3 cm column of silicagel using chloroform as the eluent. The first band of the column was the TPP byproduct. It was followed closely by a light green band of impurities. A third band, which moved very slowly, was collected and taken to dryness under vacuum on a rotary evaporator. The mixture was re-dissolved and chromatographed on a silicagel column using chloroform as eluent.

Characterization

The obtained was purified and the final product was compared with the parent free-base porphyrin-using TLC using Toluene: Hexane (1:1) as the mobile phase. UV-Visible spectra were studied with the Shimadzu spectrophotometer using Chloroform as the solvent. The FT-IR spectra was measured using KBr pellet technique with THERMONICOLET 330 and the MALDI data were obtained using a-cyano-4-hydroxy cinnamic acid as a matrix in Voyager-DE PRO instrument [6, 7, 8, 9, 10, 11].

Partition Coefficient Determination

The observed differences in the selectivity may be due to the changes of tissue penetration and distribution in the cellular mechanism[2]. Using the partition coefficient [Kp], the lipophilic character of the metalloporphyrins can be interpreted. Lipophilicity of the compounds was assessed by determining their 1-octanol/ saline buffer (pH 7.4). The known concentration of porphyrin in buffer [C_1] = 500 µg and the known concentration of porphyrin in n-Octanol [C_1] = 80 µg. A_1 is the absorbance of known concentration and A_2 is the absorbance of unknown concentration. Therefore the unknown Concentration $C_2 = A_2xC_1/A_1$. The partition coefficient was calculated according to the formula,

Kp = [compound] $_{\text{organic phase}}$ / [compound] $_{\text{aqueous phase}}$.

Table 1
Partition Coefficients of the porphyrins

S.No	Porphyrins	Standard absorbance in (lipid layer)	Concentration in lipid layer	Standard Absorbance in aqueous (buffer) layer	Concentration in aqueous (buffer) layer	Partition coefficients
1	TPP	0.1099	0.3250	0.0975	0.0941	0.4902
2	Cu (TPP)	0.0832	0.4413	0.1223	0.05189	2.000
3	Zn (TPP)	0.4253	2.2959	0.1147	0.1232	0.8132
4	Sn (TPP)	0.2786	1.2744	0.1035	1.0000	0.0757
5	Pb (TPP)	0.1799	1.7236	0.1208	0.0975	1.8990
6	T (NO_2) PP	0.1104	0.3302	0.0976	0.0981	0.4780
7	Cu (T NO_2 PP)	0.0830	0.4420	0.1232	0.05204	2.0000
8	Zn (T NO_2 PP)	0.4276	2.3042	0.1132	0.1224	0.7970
9	Sn (T NO_2 PP)	0.2790	1.2806	0.1042	1.0020	0.0760
10	Pb (T NO_2 PP)	0.1806	1.7248	0.1210	0.0964	1.9100

Photodynamic Studies On Cell Lines

The in vitro photodynamic activity of these porphyrins were analysed with human neuroblastoma cell lines (SH-SY5Y). DMSO dissolved porphyrins were treated with the cells plated in 96-well tissue culture plate. These plates were irradiated with red light for 20 minutes in UV-sterilized laminar airflow chamber and incubated for 24 hours at 37°C with 5% CO_2. Cell survival in test and standard was estimated by microscopy using trypan blue dye. Irradiation of porphyrin incubated cell culture with the IR light (Philips TL83 IR Light) for 30 minutes results a range of cell mortality. The percentage of cell death was measured by hemocytometer using trypan blue dye method (Trypan blue 0.04 % solution in 0.85 % saline) and all the steps were repeated thrice for precision [12].

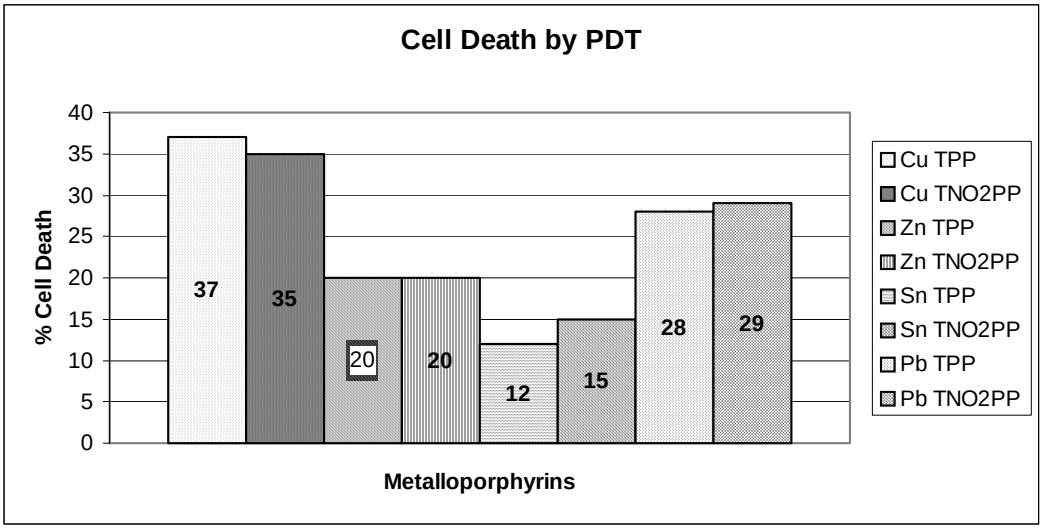
The percentage of cell death was calculated from the viable cell count and compared with the standards.

Table 2
Invitro Studies Using Neuroblastoma Cell Lines

Porphyrin 150mg/ml	Number of non-stained cells	Number of stained cells	% of viable cells	% of death cells
Standard without light	15	1	93	7
Standard without porphyrin	16	1	94	6
Cu (TPP)	9	7	56	(44 - 7) =37
Zn (TPP)	8	3	73	(27 - 7) =20
Sn (TPP)	9	2	81	(19 - 7) =12
Pb (TPP)	11	6	65	(35 - 7) =28
Cu (T NO_2 PP)	11	8	58	(42 - 7) =35
Zn (T NO_2 PP)	8	3	73	(27 - 7) =20
Sn (T NO_2 PP)	10	3	78	(22 - 7) =15
Pb (T NO_2 PP)	9	5	64	(36 - 7)=29

This was weighed against the partition coefficients of the corresponding metalloporphyrins. Finally, the percentage of cell death of the individual porphyrins was interpreted.

Graph 1
Percentage Of Cell Death Of The Individual Porphyrins



Results

Spectral Characterization

UV-Visible

Both tetra phenyl porphyrin and tetra nitrophenyl porphyrin showed an intense soret band or (B band) at 420 nm with high extinction coefficient and four Q bands in the region from 500nm to 650nm (515, 550, 590,647). The soret band transitions are due to the la_{1u} to $4e_g$ while Q bands due to $3a_{2u}$ to $4e_{g+}$.

The copper complexes of tetra phenyl porphyrin and tetra nitrophenyl porphyrin show the λmax 539 nm and 540 nm respectively. The zinc complexes of tetra phenyl porphyrin and tetra nitrophenyl porphyrin show the λmax 547 nm and 547 nm respectively. The tin complexes of tetra phenyl porphyrin and tetra nitrophenyl porphyrin show the lmax 561nm and 561nm respectively. The lead complexes of

tetra phenyl porphyrin and tetra nitrophenyl porphyrin show the λmax 653 nm and 654 nm respectively.

IR-spectral analysis

All the tetraphenylporphyrin shows a peak at 699.94 cm^{-1} is due to the presence of γ CH (meso phenyl). The short peak at 1518 cm^{-1} may be due to the presence of NH group in the porphyrin unit. Appearance of peak at 1474 cm^{-1} is due to the presence of aromatic skeleton. Peak at 1695 cm^{-1} is due to the presence of ring C=N. All the tetra nitrophenyl porphyrins show peaks around 1520 cm^{-1} and 1340 cm^{-1} that indicate the presence of nitro group. Short peaks around 1515 cm-1 may be due to NH groups and peak at 1473 cm^{-1} is due to the presence of aromatic ring.

MALDI-spectrum

Maldi spectra has been studied for the following compounds - tetra nitrophenyl porphyrin, tetra nitrophenyl copper porphyrin, tetra nitrophenyl zinc porphyrin, tetra phenyl tin and tetra phenyl lead porphyrin. All these compounds showed excellent molecular ion peaks at 794.81, 855.88, 856.90, 731.65, and 820.69 respectively.

Partition coefficient of the porphyrins

The lipophilic character varies for the individual substances and was manually calculated by performing partition coefficient using certin funnels. The Partition coefficient [(PC) = n-octanol/water (7.4 saline buffer)] of metallo derivatives Copper, Zinc, Tin, and Lead of both tetra phenyl porphyrin and tetra nitro phenyl porphyrin were calculated (see Table 1). The lipophilicity of these porphyrin derivatives was in the order of Sn < Zn < Pb < Cu.

Invitro studies using neuroblastoma

All the synthesized compounds Cu (TPP), Zn (TPP), Sn (TPP), Pb (TPP), Cu (T NO_2 PP), Zn (T NO_2 PP), Sn (T NO_2 PP), Pb (T NO_2 PP) were checked for their efficiency in PDT using neuroblastoma cell lines. These sensitizers destroy the cells by necrosis on IR irradiation (see Table 2).

Discussion

The photosensitising efficiency of the metalloporphyrins in PDT increases in the order of Cu>Pb>Zn>Sn. Among the well-characterized porphyrins and their metallic derivatives, copper and lead complexes were found to show better photodynamic activity than zinc and tin porphyrins. This methodology can be used to demonstrate the PDT procedure in a laboratory experiment.

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