Synthesis, Spectral and Biological Evaluation of Some Mesoporphyrinic Zn(II) Complexes

RICA BOSCENCU[1]*, RADU SOCOTEANU[2], MIHAELA ILIE[1], ANABELA SOUSA OLIVEIRA[4], CAROLINA CONSTANTIN[3], LUIS FILIPE VIEIRA FERREIRA[4]

[1]"Carol Davila" University of Medicine and Pharmacy, Faculty of Pharmacy ,6 Traian Vuia Str., 020956, Bucharest, Romania
[2]Romanian Academy, Institute of Physical Chemistry "Ilie Murgulescu",202 Splaiul Independentei, 060023, Bucharest, Romania
[3]„Victor Babes" National Institute for Pathology and Biomedical Sciences, Bucharest, 99-101 Splaiul Independentei, 050096, Bucharest, Romania
[4]CQFM-Centro de Química-Física Molecular and IN-Institute Nanocience and Nanotecnology, Complexo Interdisciplinar, Instituto Superior Técnico, Av. Rovisco Pais 1049-001, Lisbon, Portugal

5-(3-hydroxyphenyl)-10, 15, 20–tris-(4-carboxymethylphenyl)–21, 23 Zn(II) porphine TCMPOH$_m$Zn(II) and 5, 10, 15, 20–tetrakis-(4-carboxymethylphenyl)–21, 23 Zn(II) TCMOPZn(II) porphine were obtained through the efficient microwave-assisted methodology in order to further investigation of the theoretical and preclinical aspects of their use as sensitizers in photodynamic therapy (PDT). The complexes were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, UV-Visible spectroscopy and their structure was completely confirmed. The spectral molecular absorption properties of the porphyrinic complexes were studied in organic solvents (MeOH, EtOH, Iso-PrOH, DMSO, DMF and CH_2Cl_2), and the influence of the solvent polarity on the absorption band maxima was evaluated. Cytototoxicity in vitro preliminary studies of the compounds were also performed on the MCF7 and B16 cell lines.

Keywords: asymmetric porphyrins, Zn(II) porphyrin complexes, microvawe, FTIR, UV-Vis, NMR spectroscopy, sensitizers for photodynamic therapy, in vitro, cytotoxicity

In the recent few years, considerable attention has been focused on the development of extensively π-conjugated systems such as porphyrins and metalloporphyrins aiming at their potential application as sensitizers in photodynamic therapy (PDT).

PDT is now an established non-conventional way of treatment for a variety of oncological and non-oncological applications [1-6]. This therapy involves topical or systemic administration of the photosensitizer, followed by local application of optical doses of light of appropriate wavelength in the presence of oxygen [7, 8]. The energy of the activated photosensitiser is subsequently transferred to molecular oxygen to produce a series of energy transfers leading to the eliberation of singlet oxygen. Singlet oxygen is a very aggressive species that quickly reacts and destroy the nearby cancer cells.

The use of PDT as a cancer therapy is particularly attractive because of its fundamental specificity and selectivity [9].

The most important factor determining the efficiency PDT is how the porphyrinic compounds interacts with cells within the target tissue or tumor. The key characteristic of this interaction is the subcellular localization of the tetrapyrolic compounds which can localize within the mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus and plasma membranes [10]. The important structural features in this sense are the degree of hydrophobicity and the degree of asymmetry present in the tetrapyrolic molecules. These parameters are critical for subcellular localization of the sensitiser and dominate the efficacy of porphyrins and metalloporphyrins in PDT. So, by modifying the charge density and its distribution at the periphery of the porphyrinic macrocycle it is possible to control the route of these compounds to the target cells [11-13].

In several cases, amphiphilic porphyrin derivatives, shown their potential to be used in the treatment of tumors by PDT [14].

The research in the last decades was centered on the synthesis of asymetric porphyrins and the investigation of their structure-activity relationships. One approach to the sensitizer architecture requires the formation of asymmetrically *meso*-substituted porphyrins. Also, as the metallation of the amphiphilic porphyrins has generated a variety of photosensitisers with improved photophysical properties, our purpose was to obtain the complexes of the asymmetrically *meso*-substituted porphirins with metallic ions.

The effectiveness of these metallophotosensitisers depends largely on the nature of the coordinated central metal ion. Thus, tetrapyrroles chelated to diamagnetic metals (particularly the zinc (II)) and lanthanide ions have shown the greatest potential as photosensitizer agents [15]

Metalloporphyrins can be obtained by the classical method of complexing the porphyrinic ligands with metal salts in high-boiling nonaqueous solvents at elevated temperatures [16, 17]

Microwave-assisted reactions has been proved interesting as an alternative to classical (thermal) process because of the inherent advantages of microwave heating, which is selective, direct, rapid, internal and controllable [18-22].

Therefore, in this paper we present the synthesis of a new asymmetrical Zn(II) porphyrinic complex, 5-(3-hydroxyphenyl)-10,15,20-*tris*-(4-carboxymethylphenyl)-21,23 Zn(II) porphine and its correponding symmetrical compound 5,10,15,20-mezo-*tetrakis*-(4-carboxymethylphenyl)-21,23 Zn(II) porphine ((fig.1) using the classical method and the microwave irradiation.

* *email: rboscencu@yahoo.com*

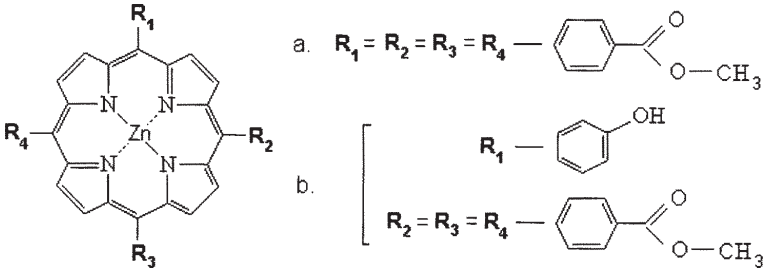

Fig. 1. Structure of Zn(II) porphyrinic complex
a) 5-(3-hydroxyphenyl)-10, 15, 20–tris-(4-carboxymethylphenyl)–21, 23 Zn(II) porphine
b) 5, 10, 15, 20–tetrakis-(4-carboxymethylphenyl)–21, 23 Zn(II) porphine

We also present the FTIR, UV-VIS and NMR characteristics of the compounds. A study of the influence of various organic solvents on the UV-VIS characteristics of the compounds is also presented.

We also performed preliminary *in vitro* cytotoxicity studies on standard cell lines to evaluate the compounds as potential pharmacutical active compounds.

Experimental part

Materials and methods

Commercially available chemicals and solvents were used as received from Sigma-Aldrich, and Merck. Porphyrinic ligands used in A method were synthesized by microwave irradiation of the corresponding substituted benzaldehyde and pyrrole [23].

IR spectra were recorded on a FT-IR 400D Nicolet Impact spectrophotometer. The substances under analysis, previously dried for 24 h , at 150°C, were processed as a spectraly pure KBr pellet. The spectra were recorded in the 4000-500 cm^{-1} spectral range.

The molecular absorption spectra were recorded on a Lamda 35 Perkin-Elmer spectrophotometer in solution, using a 10 mm path length quartz cell (Hellma), in single beam mode. UV-Vis spectra were obtained for Zn(II)TCMP and Zn(II)TCMPOH$_m$ in different solvents: MeOH, EtOH, Iso-ProOH, DMSO, DMF, CH_2Cl_2.

The porphyrin solutions were freshly prepared in the spectrally pure solvents at the concentration of $2.5x10^{-6}$ M. The solutions were kept in dark to prevent photodegradation.

The NMR spectra were recorded with a 400 MHz Bruker NMR Spectrometer. ^{1}H-NMR, ^{13}C-NMR, DEPT 90, HMQC, HMBC and COSY spectra were measured.

Viability and proliferation *in vitro* preliminary studies were performed on MCF7 (human mammary adenocarcinoma cell line – ECACC 86012803) and B16 (murine cutaneous melanoma cell line – ECACC 94042254) cells for the two complexes. The complexes were studied in two concentration ranges (3.125 - 100 μM and 0.195-6.26μM), following 2h and 24 h incubation. The compounds were tested for viability by means of the lactate dehydrogenase (LDH) release test [24], and the cell proliferation by means of the tetrazolium salt (MTS) reduction test [25].

Synthesis of the Zn(II) porphyrinic complexes

Method A

The 5, 10 ,15 ,20–meso-*tetrakis*-(4-carboxymethylphenyl)-21,23-Zn(II) porphine and 5-(3-hydroxyphenyl)-10,15,20-*tris*-(4-carboxymethylphenyl)-21,23-Zn(II) porphine were synthesized according to the previously described method [26, 27].

Solutions containing 5 mmols of tetrapyrrolic ligands (TCMP and TCMPOH$_m$) in dichloromethane were gently heated under stirring until the ligand crystals were completely dissolved. Then, several drops of 2, 6-dimethylpyridine were added together with the appropriate amount of a methanolic solution of anhydrous zinc acetate (5 mmol).

2, 6-dimethylpyridine is a Lewis base and captures the hydrogen of the porphyrin ring to produce the porphyrin dianion (P^{2-}), then the zinc ion (Zn^{2+}) is attracted by the P^{2-} to form the porphyrinic complexes.

The reaction mixture was refluxed under continuous stirring for 1 h at 55°C. The presence of the Zn (II) porphyrinic complex in the reaction mixture was monitored by UV–Vis spectroscopy and thin layer chromatography.

After cooling to room temperature, the reaction mixture was purified by several elutions on chromatographic column using dichloromethane as eluent and silica gel (100–200 mesh size) as stationary phase. The obtained yields through this method are 90 %.

These porphyrinic complexes are soluble in common organic solvents such as: methanol, ethanol, DMSO, DMF and CH_2Cl_2.

Method B (microwave-assisted synthesis method)

It is well known that microwave irradiation can significantly reduce the reaction times, side reactions, leading to increased yields, ease of purification and minimization of the amount of solvent used [18-22]. Thus, we decided to apply microwave irradiation for the synthesis of asymmetrical and symetrical Zn(II) porphyrins in order to check whether such nonclassical method of chemical activation might influence yield, selectivity and time of reaction in comparison with a classical procedure.

In order to avoid the contamination of the reaction mixture the porphyrinic complexes were prepared in the quartz vessel.

Methyl 4-formyl benzoate (0.45 mol), 3-hydroxybenzaldehyde (0.15 mol), freshly distilled pyrrole (0, 60 mol), anhydrous zinc acetate (0.15 mol), 2,6-dimethylpyridine (1 mL) and 8g of Kieselgel 60 (200-500 μm, 35-70 mesh), and dry silica were mixed at room temperature in a quartz vessel. The mixture reaction was irradiated in a microwave oven at 475W for 8 min. The extraction of samples for synthesis monitoring was performed every 2 min of irradiation. The presence of the complex in the reaction mixture was monitored by UV–Vis spectroscopy and thin layer chromatography.

After cooling, the solid product was dissolved in dichloromethane and then filtered. The solutions of the complexes in dichloromethane were concentrated by simple distillation and then the solid product was chromatographically separated on silicagel (100–200 mesh size) with dichloromethane as eluent.

The obtained yields were 68% for Zn(II)TCMPOH$_m$ and 83% for Zn(II)TCMP.

Following preparation, zinc porphyrins were characterized by IR, UV-VIS and NMR spectroscopy.

All the reactions in this paper have been successfully repeated several times with identical results and the physico-chemical features of zinc porphyrins prepared from the two different methods described above are identical.

Results and discussions

IR Spectra

The most relevant results extracted from the IR spectra of the Zn(II) complexes together with those for the corresponding free bases [23] are given in table 1.

The IR spectrum of the Zn(II) synthesized complexes and the corresponding ligands includes typical vibrational modes of both porphyrin macrocycle and phenyl substituents.

The bands at ~3310 cm^{-1} for the ligands are due to the N–H stretching of macrocycle and disappears in the metal complexes due to the replacement of central hydrogen atoms by Zn(II).

The presence of the –OH functional group in the $TCMPOH_m$ and in the corresponding complex with Zn(II) ions was confirmed by the IR spectra, where a band at ~ 3500 cm^{-1} was identified. Furthermore, the IR spectrum of porphyrinic ligands and the corresponding complex with Zn(II) ions clearly indicates the presence of the -O-CH_3 group at ~2851 cm^{-1} and C=O bands at ~ 1717 cm^{-1} .

The medium band at ~2956 cm^{-1} is attributed to the C–H stretching of the phenyl groups.

The band at 1400 cm^{-1} includes the C–H stretching of the pyrrole rings.

Table 1

CHARACTERISTIC IR VIBRATIONS OF FREE-BASE PORPHYRINS AND THEIR Zn(II) COMPLEXES

Characteristic vibration	*Wavenumber of the IR band (cm^{-1})*			
	TCMP*	**Zn(II)TCMP**	**$TCMPOH_m$***	**Zn(II)$TCMP_m$**
ν_{O-H}	-	-	3503(m)	3498(m)
ν_{N-H}	3310(w)	-	3312(m)	-
ν_{C-H}	2950(m)	2956(m)	2952(m)	2957(m)
ν_{C-H}	2923(m)	2924(m)	2923(v.s)	2922(m)
ν_{C-H} from-O-CH3	2851(s)	2851(m)	2853(m)	2851(m)
$\nu_{C=O}$	1717(v.s.)	1702(v.s.)	1718(s)	1718(s)
ν_{C-N}	1603(m)	1604(m)	1597(s)	1605(m)
$\nu_{C=N}$	1560(w)	1561(w)	1543(m)	1485(w)
$\nu_{C-H\ pyrrole}$	1400(m)	1400(m)	1401(m)	1402(m)
ν_{C-O}	1156(s)	1170(m)	1156(s)	1170(m)
δ_{C-H}	1018(m)	998(m)	1020(w)	1008(m)
$\delta_{N-H\ (pyrrole)}$	963 (m)	-	964 (m)	-
γ_{C-C}	868(w)	866(w)	858(w)	867(w)
γ C-N pyrrole	798(m)	792(m)	790(m)	794(m)

*Data for this compound were taken from [23]; the intensities of signals are: weak (w), medium (m), strong (s) and very strong (v.s.).

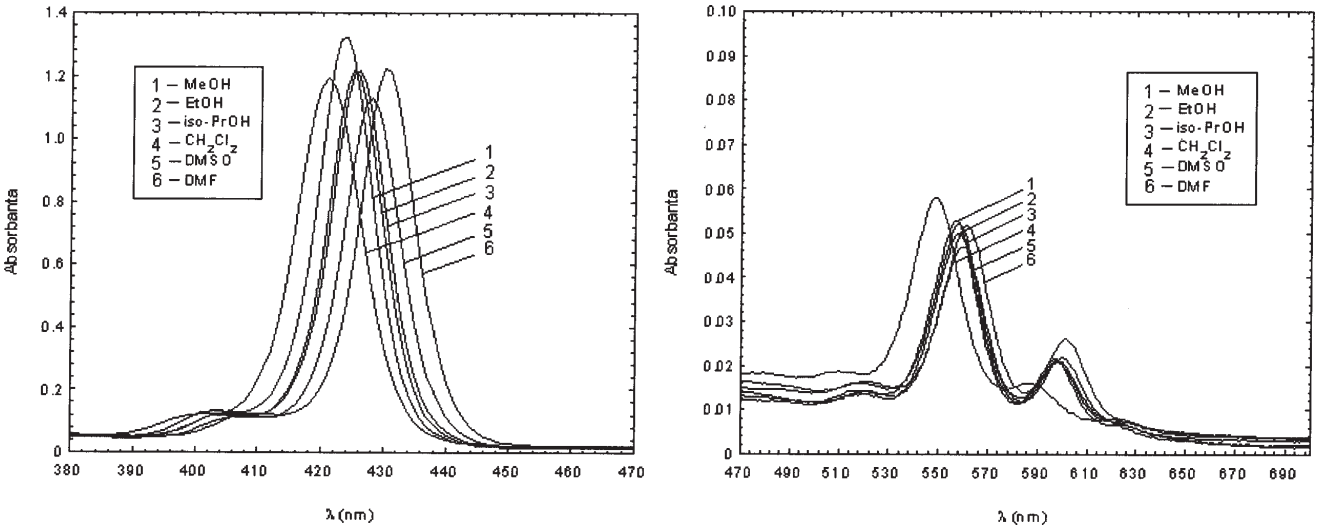

Fig. 2 Absorption spectra of the 2.5 x 10^{-6} M solutions of Zn(II)$TCMPOH_m$ in various solvents

Table 2
PARAMETERS OF THE ABSORPTION SPECTRA OF PORPHYRINIC LIGANDS AND Zn(II)TCMP, Zn(II)TCMPOH$_m$ IN VARIOUS SOLVENTS (c=2.5x10^{-6}M)

Solvent	λ_{max} (nm) [lgε] (L mol^{-1} cm^{-1})				
	Soret Band	Qy(1,0)	Qy(0,0)	Qx(1,0)	Qx(0,0)
5, 10, 15, 20-meso-*tetrakis*-(4-carboxymethylphenyl) - 21,23-H porphine*					
MeOH	415.3 [5.528]	514.8 [4.415]	544.3 [4.387]	591.9 [4.265]	646.9 [4.342]
EtOH	416.4 [5.546]	512.5 [4.428]	547.4 [4.283]	590.4 [4.225]	647.4 [4.146]
Iso-PrOH	416.8 [5.511]	513.5 [4.301]	546.9 [4.049]	592.4 [3.857]	648.4 [3.602]
CH_2Cl_2	419.6 [5.699]	515.2 [4.344]	550.0 [4.000]	590.1 [3.806]	645.5 [3.643]
DMSO	420.9 [5.602]	515.6 [4.310]	550.0 [4.049]	590.1 [4.000]	645.6 [3.833]
DMF	419.3 [5.662]	514.3 [4.326]	548.7 [4.017]	589.6 [3.833]	645.3 [3.716]
5, 10 ,15 ,20–meso-*tetrakis*-(4-carboxymethylphenyl)-21,23-Zn(II)porphine					
MeOH	424.0 [5.591]	-	557.4 [4.146]	597.3 [3.806]	-
EtOH	425.6 [5.488]	-	558.6 [4.225]	598.0 [4.049]	-
Iso-PrOH	425.7 [5.342]	-	557.9 [4.301]	597.0 [4.193]	-
CH_2Cl_2	421.4 [5.614]	-	548.8 [4.274]	588.0 [3.778]	-
DMSO	430.9 [5.634]	-	559.8 [4.428]	601.5 [4.000]	-
DMF	428.0 [5.560]	-	559.5 [4.158]	599.7 [3.827]	-
5- (3-hydroxyphenyl)-10, 15, 20–*tris*-(4-carboxymethylphenyl) – 21,23-H porphine*					
MeOH	422.4 [5.582]	513.1 [4.158]	554.8 [4.193]	595.5 [3.924]	647.6 [3.643]
EtOH	424.1 [5.561]	513.4 [4.134]	556.3 [4.158]	596.1 [3.857]	649.4 [3.556]
Iso-PrOH	424.6 [5.577]	513.1 [4.146]	556.0 [4.182]	596.7 [3.881]	650.3 [3.602]
CH_2Cl_2	420.1 [5.607]	515.2 [4.121]	548.8 [4.146]	588.9 [3.716]	647.9 [3.556]
DMSO	429.3 [5.546]	516.1 [4.170]	558.6 [4.182]	598.8 [3.944]	648.8 [3.681]
DMF	426.5 [5.519]	514.9 [4.107]	556.8 [4.121]	597.9 [3.833]	648.2 [3,556]
5-(3-hydroxyphenyl)-10, 15, 20-*tris*-(4-carboxymethylphenyl)-21,23- Zn(II)porphine					
MeOH	423.6 [5.725]	-	556.8 [4.326]	597.6 [3.924]	-
EtOH	425.4 [5.695]	-	558.0 [4.301]	598.5 [3.924]	-
Iso-PrOH	426.1 [5.688]	-	558.3 [4.318]	597.9 [3.924]	-
CH_2Cl_2	421.0 [5.677]	-	548.5 [4.365]	586.2 [3.806]	-
DMSO	430.5 [5.688]	-	561.3 [4.318]	601.8 [4.017]	-
DMF	427.9 [5.654]	-	559.5 [4.274]	599.7 [3.944]	-

**Data for this compound were taken from [28]; MeOH- methanol, EtOH-ethanol, Iso-PrOH-isopropyl alchol, DMSO - dimethylsulfoxide, DMF-dimethylformamide, CH_2Cl_2-dichloromethane*

Molecular electronic spectra

UV-Vis spectra were obtained for Zn(II)TCMP and Zn(II)TCMPOH$_m$ in various solvents (MeOH, EtOH, Iso-ProOH, DMSO, DMF, CH_2Cl_2), the main results being presented in figure 2 (for Zn(II)TCMPOH$_m$) and table 2.

The absorbtion spectra of each compound undergo distinctive changes depending on the solvent type. Considering that the values of the refractive index (n_D^{20}) of the solvents used in is in a close range (from 1.3290 for Me-OH to 1.4758 for DMSO) focused on the increasing polarity of the solvents.

In these conditions the electronic absorption spectra of free bases display a typical pattern in the visible region, with an intense Soret band, followed by the four Q-bands: Qy(1,0), Qy(0,0) , Qx(1,0), Qx(0,0), which are monotonously decreasing in intensity.

Taking into account the fact that UV-Vis spectra of porphyrins are extremely sensitive to such processes as metallation, our interest focussed on 5-(3-hydroxyphenyl)-10,15,20–*tris*-(4-carboxymethylphenyl)–21,23Zn(II) porphine and 5,10,15,20–*tetra*-(4-carboxymethylphenyl)–21,23 Zn(II) porphine, that display a high symmetry (D_{4h}); as a consequence, the four previous Q bands reduce themselves to two.

The main observation is that the wavelength of the Soret band has a very sensitive response to the tested solvents. The bathochromic shift of the Soret band follows the order CH_2Cl_2 > MeOH > >EtOH > Iso-ProOH > DMF > DMSO. The same order is observed in the Q bands. The two metallated structures proved to display a similar sensitivity to solvent change in terms of the Soret band displacement (table 2). The consequences of the solvent polarity are less observed on the Q bands region. From the free base influence point of view, the shift is less significant.

NMR Experiments

In this study, various NMR experimental approaches (including ^{13}C-NMR, DEPT 90, DEPT 135, COSY, HMBC, and HMQC) were developed.

We present below the chemical shifts and the multiplicities of the ^{1}H-NMR signals for Zn(II) complexes and other important adjacent data provided by ^{13}C-NMR.

The comparative analysis of NMR data corresponding to the ligand [23] and their Zn(II) complexes shows the lack of ^{1}H-NMR signal corresponding to the pyrrole NH-type protons from the zinc complex NMR spectra, thus attesting the coordination of the metal ion to the nitrogen atoms of the porphyrinic macrocycle.

Zn(II)TCMP

^{1}H-NMR: 4.12 (12H, s, O-CH_3), 8.44 (8H, d, H_o), 8.45 (8H, d, $H_{\beta pyrr}$), 8.55 (8H, d, H_m).

^{13}C-NMR: 52.4 ($C_{O\text{-}CH3}$), 119.4 ($C_{5,10,15,20}$), 128.9, 129.6, 134.5($C_{\beta pyrr}$), 146.5 ($C_{\alpha pyrr}$), 166.9(C_{COO}).

Zn(II)TCMPOH$_m$

^{1}H-NMR: 4.13 (9H, s, O-CH_3), 5.19 (1H, s, OH), 7.01 (1H, s, H), 7.61 (1H, t, H), 7.64 (1H, d, H), 7.61 (1H, m, H), 8.78 (6H, d, $H_{\beta pyrr2}$), 8.99 (8H, d, $H_{\beta pyrr1}$).

^{13}C-NMR: 53.6 ($C_{O\text{-}CH3}$), 114.8, 120.0, 121.3 (C_5), 122.0 ($C_{10,15,20}$), 127.9, 128.23, 129.9, 134.5 ($C_{\beta pyrr}$), 135.4, 145.2, 146.8, 150.9 ($C_{Phenyl\text{-}OH}$), 167.8 (C_{COO}).

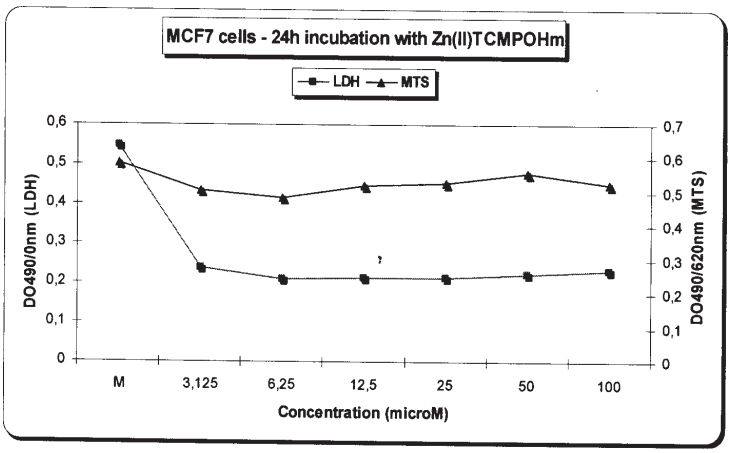

Fig. 3 Cellular response of the MCF7 cells folowing 24h incubation with Zn(II)TCMPOH$_m$

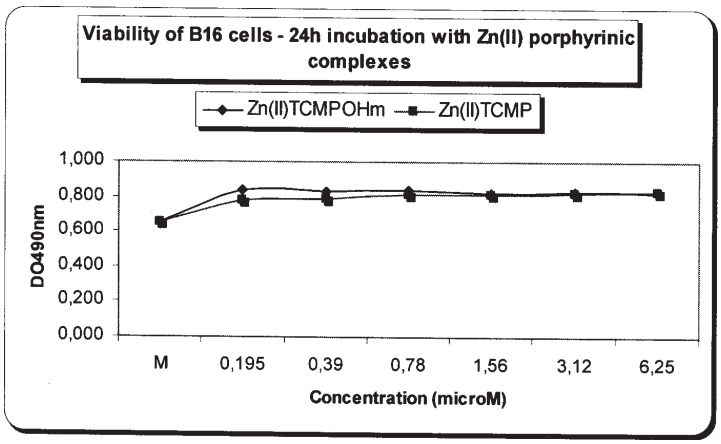

Fig. 4 Cellular viability of B16 cells lines following 24h incubation with Zn(II) porphyrinic complexes

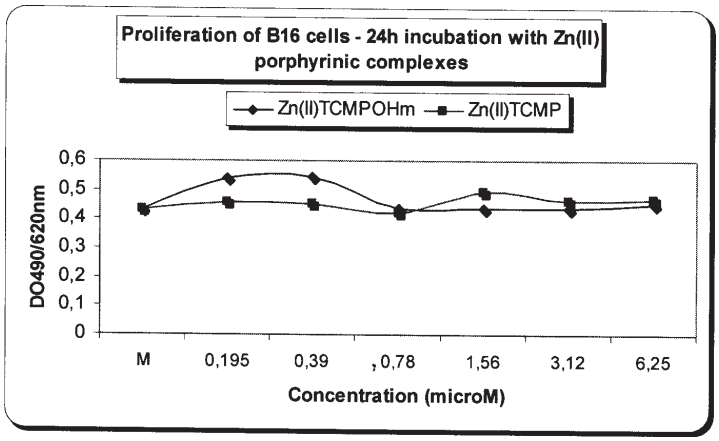

Fig. 5 Proliferative capacity of B16 cells folowing 24h incubation with Zn(II) porphyrinic complexes

In vitro cytotoxicity studies

Viability and cell proliferation capacity of MCF7 cells in the presence of Zn(II)TCMP and Zn(II)TCMPOH$_m$ in concentrations ranging in the fields 100μM-3.125μM and 6.25μM-0.195μM was studied, as wel as that of B16 cell in the concentration range 6.25μM-0.195μM, for 2 and 24 h incubation with the compounds.

The results obtained indicate the fact that for MCF7 cells following 24h of incubation with Zn(II)TCMPOH$_m$ at the concentration 3.125 μM tends to exert a slight effect on the proliferative capacity (fig. 3). For the 6.26 -0.195 μM of complexes, a low basal cytotoxicity of the compounds could be found.

It is generally known the resistence of melanoma to classic antitumour therapy (radiotherapy), so the research is directed towards the identification of certain agents that should sensitize this kind of tumours. The results on B16 cels following 24 h incubation is characterised by a slight decrease in the MTS reduction and an increase of the LDL release. (fig. 4 and 5).

The preliminary studies attest the low or inexistent cytotoxicity of the complexes Zn(II) TCMP and Zn(II)TCMPOH$_m$ on the studied cells and concentrations.

Conclusions

In this paper, we present a convenient and rapid method the synthesis of asymmetrical and symmetrical Zn (II) porphyrins using microwave irradiation. The synthesized compounds were 5-(3-hydroxyphenyl)-10,15,20-*tris*-(4-carboxymethylphenyl)-21,23 Zn(II) porphine (Zn(II)TCMPOH$_m$) and its corresponding symmetrical compound 5,10,15,20-mezo-*tetrakis*-(4-carboxymethyl-phenyl)-21,23 Zn(II) porphine (Zn(II)TCMP). For this particular pair of Zn(II) metalated porphyrins the microvawe assisted synthetic method, at this stage of development, proved to be inferior to the classical method in terms of reaction yield but faster and easier to handle. The compounds obtained by the two methods (classical and

microwave irradiation) presented the same spectral characteristics (FTIR, UV-Vis and NMR), what demonstrate that they are identical as structures.

The UV-VIS spectra performed on the compounds dissolved in different solvents indicated a slight bathochromic shift with increasing solvent polarity of both the Soret and the Q bands.

Preliminary *in vitro* cytotoxicity studies revealed a low to inexistent toxicity exerted by the two compounds on both MC7 (human mammary adenocarcinoma) and B16 (murine cutaneous melanoma) cell lines as evidentiated from viability and proliferation standard tests.

Acknowledgements: This work was supported by National Research Project PN II 41-047/2007-2010 (CELLPHARM)

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Manuscript received: 5.11.2008