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Photosensitizing action of isomeric zinc *N*-methylpyridylporphyrins in human carcinoma cells

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Abstract

The success of photodynamic therapy (PDT), as a minimally invasive approach, in treating both neoplastic and non-neoplastic diseases has stimulated the search for new compounds with potential application in PDT. We have previously reported that Zn(II) *N*-alkylpyridylporphyrins (ZnTM-2(3,4)-PyP^{4+} and ZnTE-2-PyP^{4+}) can act as photosensitizers and kill antibiotic-resistant bacteria. This study investigated the photosensitizing effects of the isomers of ZnTMPyP^{4+} (ZnTM-2(3,4)-PyP^{4+}) and respective ligands on a human colon adenocarcinoma cell line. At 10 μM and 30 min of illumination the isomeric porphyrins completely inhibited cell growth, and at 20 μM killed approximately 50% of the cancer cells. All these effects were entirely light-dependent. The isomers of the ZnTMPyP^{4+} and the respective ligands show high photosensitizing efficiency and no toxicity in the dark. Their efficacy as photosensitizers is comparable to that of hematoporphyrin derivative (HpD).

Keywords: *Photosensitizers, cancer, photodynamic therapy, isomeric zinc methylpyridylporphyrins, isomeric* N*-methylpyridylporphyrins*

Abbreviations: *ZnTM-2-PyP^{4+},* ortho-isomer*, Zn(II) tetrakis(*N*-methylpyridinium-2-yl); ZnTM-3-PyP^{4+},* meta-isomer*, Zn(II) tetrakis(*N*-methylpyridinium-3-yl)porphyrin; ZnTM-4-PyP^{4+},* para-isomer*, Zn(II) tetrakis(*N*-methylpyridinium-4-yl); H$_2$TM-2(3,4)-PyP^{4+},* ortho*(2)-,* meta*(3)- and* para*(4)-isomers of metal free ligands; PDT, photodynamic therapy; HpD, hematoporphyrin derivative; ANOVA, analysis of variance*

Introduction

Photodynamic therapy (PDT) is a relatively new, minimally invasive therapeutic approach for the management of both neoplastic and non-neoplastic diseases [1,2]. It is based on the combined use of a light-absorbing compound (photosensitizer), and irradiation with visible light of a wavelength(s) matching the absorption spectrum of the photosensitizer [3], whereby, $^1O_2$ and/or $O_2^{\cdot-}$ are produced, the latter giving rise to H_2O_2. Unlike $O_2^{\cdot-}$ and H_2O_2, however, $^1O_2$ has no known enzymatic scavengers [4]. The main classes of cytotoxic reactions are photooxidation by singlet oxygen, photooxidation by radicals, and photoreaction not involving oxygen. Ideally, a photosensitizer should be: (a) a pure compound with constant composition; (b) not toxic in the absence of light; (c) enriched in the targeted tissue; (d) rapidly eliminated from the body to avoid systemic toxicity; (e) of a high quantum yield; (f) of a high molar absorptivity in 600–800 nm range, where tissue penetration of light is at maximum and the light energetic enough to produce singlet oxygen [5].

Most photosensitizers possess a heterocyclic ring structure similar to chlorophyll or heme. In general,

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they may be divided in three broad families: (a) porphyrin-based compounds; (b) chlorophyll-based compounds and (c) dyes [6]. Porphyrins, developed as photosensitizers in the 1970s and early 1980s, are the first generation of PDT agents with a prominent member hematoporphyrin D (HpD, photofrin). Although highly effective, HpD has a number of disadvantages including a tendency to cause prolonged skin photosensitivity and a poorly defined chemical composition, which makes understanding of its mode of action and pharmacokinetics difficult [7]. Thus, a second generation of photosensitizers [5,8–13], most of which are porphyrin-based molecules, have been developed to improve the selectivity of the uptake into the target cells and to increase the depth of the penetration with light of longer wavelengths than that used to activate photofrin.

Porphyrin structure offers almost limitless possibilities for modifications, which can affect the uptake, the distribution and the photochemistry of the photosensitizer. For example, the tetra-cationic porphyrinic ligand bearing *para*-methylpyridyl, at *meso* positions (H_2TM-4-PyP) is taken up faster by human skin fibroblasts and localizes in the nucleus, while the hexylpyridyl analogue (H_2TnHex-4-PyP) (charges are omitted throughout the text) entered the cells much more slowly and was found mainly in the cytosol [14]. The distribution of the methyl derivative (H_2TM-4-PyP) in BALB/c mice bearing MS-2 fibrosarcoma has also been studied [15]. A peak tumor concentration was reported 24 h post injection, and at that time no H_2TM-4-PyP was detected in blood and skin [15]. We have previously reported [16] that Zn(II) *N*-alkylpyridyl-porphyrins (ZnTM-2(3,4)PyP and ZnTE-2-PyP) can act as photosensitizers with the ability to kill antibiotic-resistant pathogenic bacteria. They were more efficient than HpD in causing NADH oxidation, photodynamic growth inhibition and photodynamic kill of *Escherichia coli* [16]. No data about the distribution of ZnTMPyP in cultured mammalian cells or in animals are available, but Mn(III) porphyrin pentacationic analog was found to associate with mitochondria [17]. Based on the inability of the Zn site to be redox active and thus unable to affect the cellular redox status and cellular signaling, Zn porphyrins are expected not to be toxic in the dark. Thus, Zn substituted porphyrins represent a promising group of photosensitizers, which, if properly designed, might be able to fulfill most of the requirements for the photodynamic treatment of neoplastic and non-neoplastic diseases. In an attempt to elucidate the photosensitizing activities of Zn porphyrins, we have investigated the effects of isomeric ZnTMPyP and the respective metal-free ligands on the growth and survival of cultured cancer cells.

Materials and methods

H_2TM-(2,3,4)-PyP ligands and HpD were obtained from MidCentury Chemicals (Chicago, IL) and were used without further purification. The Zn(II) *N*-methylpyridylporphyrins were prepared as described previously [16]. The structures of the *ortho*, *meta* and *para* Zn-containing isomers are shown in Figure 1.

Human colon adenocarcinoma LS174T cell line was kindly provided by Dr Christopher Ford (Faculty of Medicine, Kuwait University). The cells were grown in minimal essential medium (MEM), (Gibco BRL) supplemented with 10% fetal bovine serum, 1.2% L-glutamine, 1.2% MEM non-essential amino acids and 1.2% penicillin/streptomycin. Cells were incubated at 37°C under 5% CO_2 saturation and used for experiments at ~90% confluence. The cells were plated on 96 well plates at a density of about 5×10^3/well and incubated overnight to adhere. Sterile solutions of the photosensitizers were added at concentrations of 5, 10, 20 or 50 μM and 30 min prior to illumination of the plates. Two TL8W Philips white fluorescent tubes mounted on a light box with a white translucent screen providing a fluence rate of 0.5 mW/cm^2 were used. The cells were illuminated for 10, 20, 30, 40 and 60 min at 25°C. No measurable increase of the temperature was observed during the period of illumination. The light controls (without photosensitizers) and dark controls (containing the same concentrations of the respective photosensitizers, but kept in the dark for the time of the illumination) were analyzed in parallel. At the indicated time intervals after the illumination, cell viability was determined using trypan blue exclusion. The cell growth was followed for up to 3 days after the illumination.

The leakage of ATP from cells was determined for controls, ZnTMPyP-treated in the dark, and ZnTMPyP-illuminated groups (all at 7.5×10^3 cells/well). Immediately after the illumination,

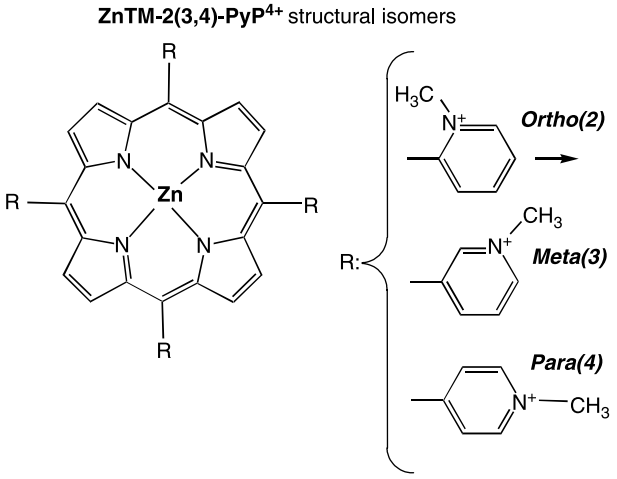

Figure 1. Structural isomers of Zn(II) tetrakis *N*-methylpyridylporphyrins.

50 μl samples of the medium were transferred into eppendorf tubes and centrifuged for 3 min at 4°C. Aliquots (25 μl) of the supernatant were taken and ATP was measured with an ATP Bioluminescent Assay Kit (Sigma) according to the manufacturer's instructions.

All experiments were repeated at least three times with 3–5 replicates. The mean ± SEM are presented. Statistical analysis was performed using ANOVA. $p < 0.005$ was considered statistically significant.

Results

Optimal illumination time

The initial aim of this investigation was to optimize the conditions for illumination and the concentrations of the Zn porphyrins in order to achieve killing of approximately 50% of the tumor cells. For this purpose one of the isomers, *meta* ZnTM-3-PyP, was selected and tested over a concentration range of 5–50 μM for the effects on the cell viability after various light exposure times. Figure 2 demonstrates that 20 μM of ZnTM-3-PyP, within 30 min of illumination, killed about 50% of the cells. For this reason, 30 min was selected as the time of light exposure in the subsequent experiments.

Photodynamic growth inhibition

Figure 3 shows the effect of varying concentrations of ZnTM-3-PyP on the growth of cancer cells. The cell division was completely inhibited by 10 μM of the compound after 30 min of illumination. This effect was entirely light-dependent since no growth inhibition was observed when cultures treated with up to 50 μM of ZnTM-3-PyP were grown in dark (data not shown).

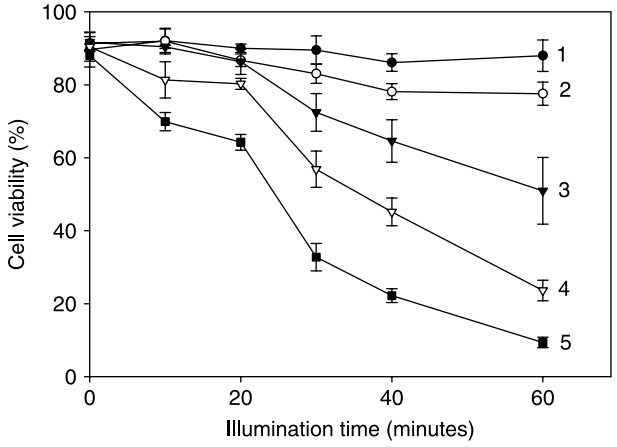

Figure 2. Time and concentration dependence of the photodynamic loss of viability. LS174T cells were plated at a density of 1.16×10^3 cells/well on a 96 well plate. After addition of ZnTM-3-PyP to give the indicated concentrations, the plate was illuminated. The cell viability was expressed as a percentage of viable cells (trypan blue exclusion) relative to the total number of cells, which was taken as 100%. Line 1, control; line 2, 5 μM ZnTM-3-PyP; line 3, 10 μM ZnTM-3-PyP; line 4, 20 μM ZnTM-3-PyP; line 5, 50 μM ZnTM-3-PyP.

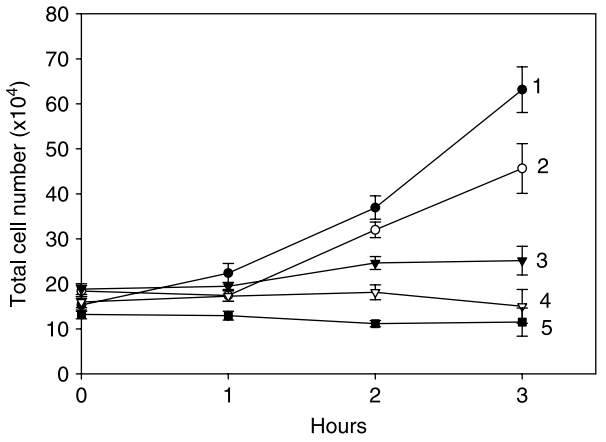

Figure 3. Effect of ZnTM-3-PyP^{4+} on cell growth. LS174T cells were plated on a 96 well plates and allowed to attach. After addition of ZnTM-3-PyP at the indicated concentrations, the plates were illuminated. The plates were maintained at 37°C and 5% CO_2, and the total number of cells in five wells was counted at the indicated time intervals. The respective controls containing the same concentrations of ZnTM-3-PyP were grown under the same conditions but in the absence of illumination. Line 1, control; line 2, 5 μM ZnTM-3-PyP; line 3, 10 μM ZnTM-3-PyP; line 4, 20 μM ZnTM-3-PyP; line 5, 50 μM ZnTM-3-PyP.

Time-dependence of the photo-induced loss of viability

In order to determine if cells that survive the illumination period can recover, or if the photodynamic treatment triggers irreversible cell damage leading to cell death at a later time, we investigated the time-dependence of the photo-induced loss of viability. As demonstrated in Figure 4, even when illuminated in the presence of low ZnTM-3-PyP concentration (5 μM), where no large initial loss of viability was observed, the

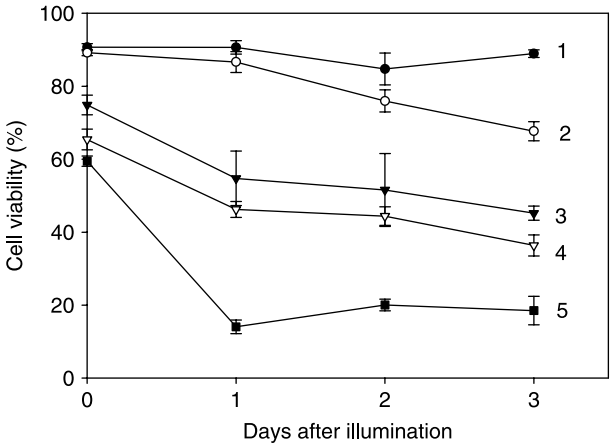

Figure 4. Time-dependence of the photo-induced loss of viability. All conditions were as described for Figure 2, except that the cell viability was assayed immediately after the illumination period, and then 1, 2 and 3 days later. Line 1, control; line 2, 5 μM ZnTM-3-PyP; line 3, 10 μM ZnTM-3-PyP; line 4, 20 μM ZnTM-3-PyP; line 5, 50 μM ZnTM-3-PyP.

percentage of viable cells progressively decreased over the 3-day observation period. This indicates that apart from the direct photokill, illumination inflicted irreparable damage, which was manifested as delayed loss of viability. This effect was best demonstrated with 50 μM ZnTM-3-PyP. At this concentration, the viability loss was approximately 50% immediately following the illumination period, and reached almost 100% after 1 day. Another possible explanation is that medium nutrients are a target of photosensitized oxidation producing toxic species in the medium. Thus, the longer the cells are kept in such a toxic environment, the higher the loss of viability. The loss of viability was not due to the toxicity of the ZnTM-3-PyP itself, because no loss of viability was observed in parallel cultures kept in the dark.

Comparison of Zn(II) N-*methylpyridylporphyrins and their respective free ligands*

Figure 5A–C shows a dose-dependent photo toxicity of the cationic *N*-methylpyridylporphyrins and their Zn(II) complexes. For all three isomers, the phototoxicity increased in the order: HpD > ZnTMPyP > H_2-TMPyP. The differences in the photosensitizing abilities of the tested compounds were, however, relatively small and were significant only at the lower concentration range of 10–20 μM. At 50 μM, ZnTMPyP and HpD were equally efficient, while the free ligands showed weaker activity over the entire concentration range.

Comparison between ortho-, meta- *and* para-*isomers*

The illumination of the cell suspensions in the presence of *N*-methylpyridylporphyrins or their Zn(II) complexes caused a concentration-dependent loss of viability (Figure 6A and B). For the Zn(II) *N*-methylpyridylporphyrins, pronounced differences in the phototoxicity of the isomers were observed only at concentration of 10 μM, with *ortho* > *meta* = *para* (Figure 6A). At higher concentrations, all three isomers were equally efficient as photosensitizers, being practically equal to HpD at 50 μM. In contrast, the *meta* ligand was least effective at 10 μM, while *ortho* and *para* demonstrated equal phototoxicity (Figure 6B). At higher concentrations, the *para*-isomer became more effective, and phototoxicity was equal to HpD at 50 μM.

Leakage of ATP

It has been reported that reduction in tumor ATP levels appears to be an early biochemical response

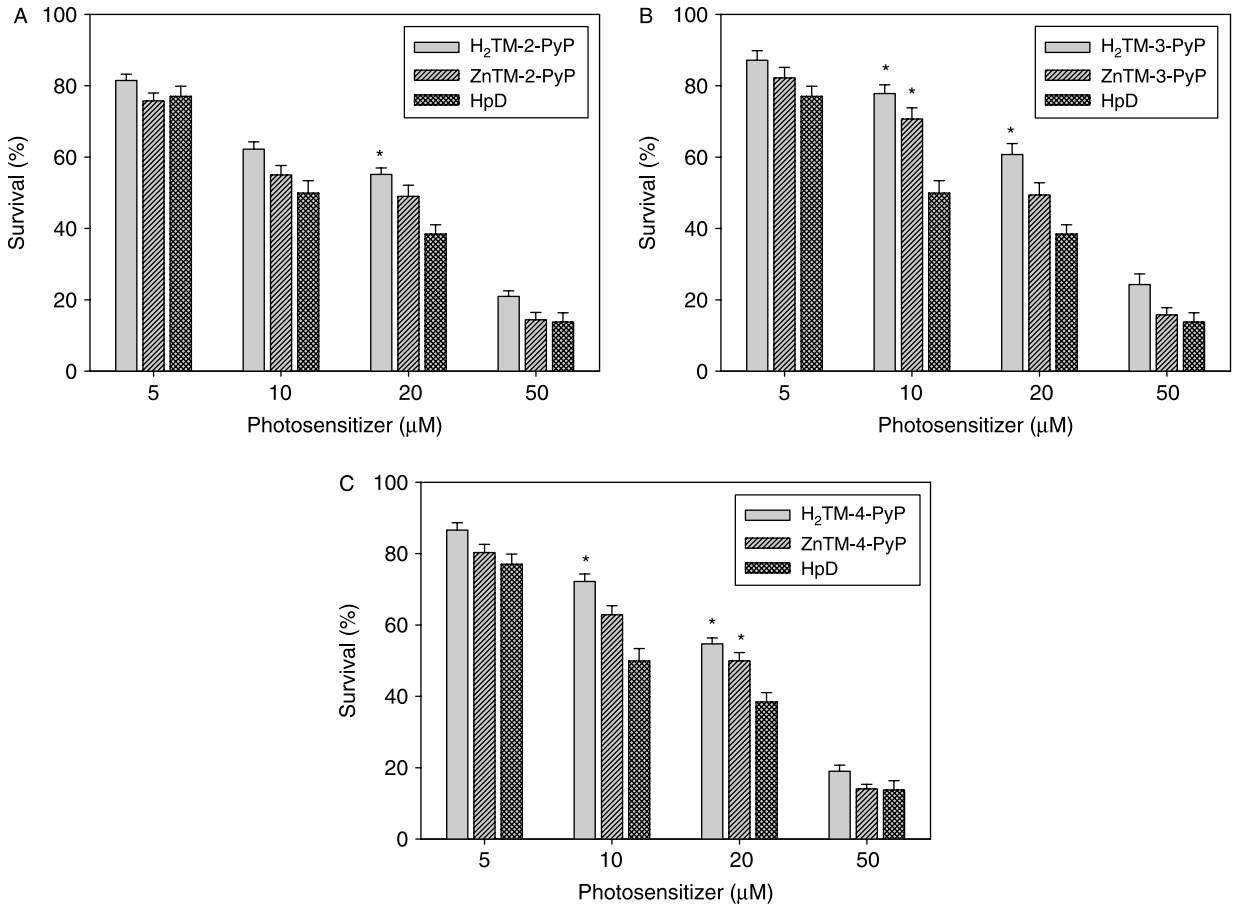

Figure 5. Comparison among the free ligands, Zn(II) complexes and HpD. All conditions were as described for Figure 2, except that all photosensitizers were applied at 20 μM. Cell viability was assessed 3 h after illumination. Panel A, *ortho*-; panel B, *meta*- and panel C, *para*-isomers of H_2TMPyP and ZnTMPyP were tested. *$p < 0.005$ compared to HpD.

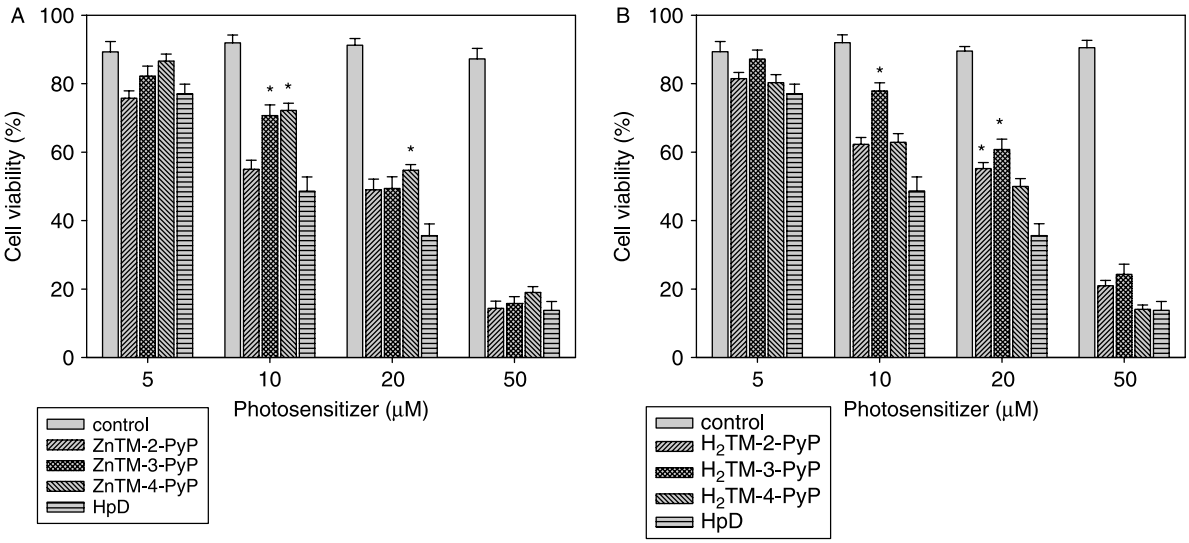

Figure 6. Comparison among the *ortho*-, *meta*- and *para*-isomers of ZnTMPyP and H_2TMPyP. Cells were treated as described for Figure 2. Cell viability was assessed 3 h after illumination. Panel A, ZnTMPyP; panel B, H_2TMPyP. $*p < 0.005$ compared to HpD.

to PDT [18]. The leakage of ATP might be among the reasons for a rapid depletion of ATP in photo-treated tumor cells. We found that a dramatic loss of ATP takes place during the illumination of the photosensitized cells. Thus, immediately after 30 min of illumination, the ATP content in the medium of the controls was 12.00 ± 3.77 pmoles/10^3 cells, against 139.73 ± 15.96 pmoles/10^3 cells for the ZnTM-3-PyP-treated cells. The leakage was due to the photo-induced damage, since no ATP leakage (medium content 12.20 ± 0.82 pmoles of ATP/10^3 cells) was observed in the cells treated with the same concentration of ZnTM-3-PyP, but not exposed to light.

Discussion

Among the photosensitizers in clinical use, and those under development, the majority belong to the porphyrin family. The reason lies in the almost limitless possibilities of core structure modification that offer great opportunities to develop an ideal photosensitizer. It was the aim of this study to further explore the potential of porphyrinic structures, particularly cationic ones, to photo-induce cancer cell killing.

The photochemistry of the *N*-methylpyridylporphyrins and their Zn(II) complexes has been studied previously [19]. No significant differences among *ortho*-, *meta*- and *para*-isomers of Zn complexes were reported with respect to fluorescence. The triplet state yields are high (>0.85) for all ligands and Zn(II)-containing isomers [19]. In general, the higher the triplet state yield, the higher is the production of $^1O_2$, which means the higher the efficiency of the photosensitizer. The triplet state lifetimes for the free ligands are in the range 1.16–0.17 ms and decrease along the series *ortho* > *meta* > *para* [19].

Introduction of zinc increases the triplet lifetimes to 1.4 ms for ZnTM-2-PyP and to 2.0 ms for the *meta*- and *para*-isomers [19]. It might be expected, therefore, that in our experiments there would be differences with respect to cell damage between the ligands and their Zn complexes, and also among the isomers of Zn porphyrins and their respective ligands. Indeed, our results show that for the LS174T cell line, all isomers of the Zn complexes were more phototoxic than the respective free ligands. Among the Zn(II) complexes, the *ortho*-isomer (ZnTM-2-PyP) was the most efficient, but only at a relatively low concentration (10 μM). The differences between the isomers of the Zn complexes practically disappeared when the compounds were applied at the highest concentration (50 μM). At such a concentration, the amounts of photosensitizers accumulated in the cells seems to compensate for the slight differences in their photoefficacy. Because the photochemical differences among the compounds tested are small, their phototoxicity would depend mainly on their cellular uptake and tissue distributions. Some other actions of Zn porphyrins may also account for the effects observed, and they as well as the distribution of the compounds, will be explored in our future studies. Importantly, neither ligands nor Zn porphyrins showed toxicity in the dark.

The slight advantage of HpD over the ZnTMPyP at lower concentrations is most probably due to its higher hydrophobicity (and possibly to the anionic charge) compared to the Zn porphyrins, which facilitates its penetration into the cells [20]. Another factor that may contribute is differences in the light absorption [21].

Even though the spectral characteristics of HpD and ZnTMPyP do not differ considerably [16], their spectral properties may change when taken up by the cells [22].

A noteworthy finding of this study is the photo-induced leakage of ATP from the cells. Such an effect has been reported for the photodamaged yeasts [23]. It is probable that rapid and significant membrane damage takes place when cells are illuminated in the presence of ZnTMPyP. Photo-induced ATP loss may result directly from membrane damage or from activation of channels such as those reported to mediate ATP release in response to mechanical or osmotic stress, or hypoxia [24–26]. Even if mitochondria remain intact during the photo-treatment, such a leakage would inevitably lead to ATP depletion and finally to cell death.

Conclusions

Our study demonstrates that isomeric zinc *N*-methylpyridylporphyrins are as effective as HpD with respect to their ability to photokill cancer cells *in vitro*. Differences in the activity among the isomers are small and only apparent at lower concentrations. Since the compounds were non-toxic in the dark, and are expected, by analogy with the behavior of the free ligands, to accumulate in tumors, but not in the skin [15], zinc *N*-methylpyridylporphyrins appear promising as a group of photosensitizers with potential application for *in vivo* PDT. Further studies are needed to evaluate their cellular uptake and distribution, as well as their effects and organ distribution *in vivo*.

Acknowledgements

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