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Research Article

BIOLOGICAL EVALUATIONS OF PROTOPORPHYRIN IX, PHEOPHORBIDE *a*, AND ITS 1-HYDROXYETHYL DERIVATIVESS FOR APPLICATION IN PHOTODYNAMIC THERAPY

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ABSTRACT

Protoporphyrin IX **(1)**, pheophorbide *a* **(3)**, and its 1-hydroxyethyl derivativess **(2,4)** were studied *in vitro* as photosensitizer candidates for photodynamic therapy. Protoporphyrin IX has been indicated to have a cytotoxic effect in the absence of light excitation. The dark toxicity of **1-4** was evaluated against normal cells (Vero), human epithelial cervix carcinomas (HeLa) and human breast cancer (T47D) cell lines, while the phototoxicity of **1-4** was evaluated against HeLa and T47D cell lines. Moreover, the MTT assay was employed to evaluate cell viability. The 1-hydroxyethyl derivativess showed a lower dark toxicity in the three types of cells compared to the parent molecules. It was also observed that the parent molecules were more phototoxic than those of its 1-hydroxyethyl derivativess.

Keywords: Protoporphyrin IX, Pheophorbide *a*, 1-hydroxyethyl derivativess, Photodynamic therapy.

INTRODUCTION

Photodynamic therapy (PDT) can be defined as the administration of a non-toxic drug or dye known as a photosensitizer (PS) either systemically, locally, or topically to a patient bearing a lesion (frequently but not always cancer). This is followed by the illumination of the lesion with visible light, usually a long wavelength red light, which leads to the generation of cytotoxic species in the presence of oxygen and consequently to cell death and tissue destruction[1]. The ideal PS should exhibit a low level of dark phototoxicity and systemic toxicity, a good tumor selectivity and should simultaneously avoid accumulation in the surrounding healthy tissues and be rapidly eliminated from an organism to prevent prolonged photosensitivity[1,2]. In other cases, there is also an increase in effort to discover new anti-cancer with no-cytotoxicity to the normal cells[3].

Hematoporphyrin derivativess (HPD) was the first PS identified, and reports of selective localization of porphyrins in tumors appeared until the 1960s[1]. However, there is always the possibility of PS uptake by normal cells which can cause collateral damage in dark conditions. Therefore, PS should exhibit high phototoxicity with no dark toxicity.

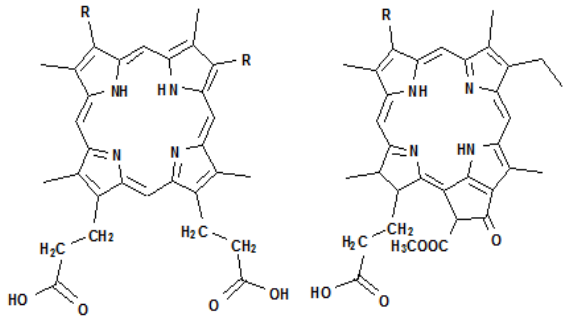
Some PS can easily be prepared by partial synthesis using abundant natural starting materials, such as heme or chlorophyll. This route leads to both economical and environmental advantages compared to complicated total chemical synthesis[4]. Protoporphyrin IX (**1**) and pheophorbide *a* (**2**) are chemical derivativess obtained from naturally occurring porphyrins and chlorins, and both compounds have been studied as PS for PDT.

The anti-tumor effect of a protoporphyrin IX-based PDT has been successfully demonstrated in a wide range of human malignant cell lines[5-8]. However, Chu *et al.* indicated the cytotoxic effect of 5-aminolevulinic acid (ALA) treatment on lymphocytes without light excitation[9]. Lymphocytes are blood cells that circulate around the whole body, meaning that they have a greater chance than other non-blood cells to encounter drug molecules that are delivered to the tumor. Furthermore, Koningsberger *et al.* showed that protoporphyrin IX at a concentration of 0.5–100µg/ml inhibited cellular proliferation in hepatocellular carcinoma cell lines under dark conditions[10].

Pheophorbide *a,* a chlorin compound, has been shown previously to be a good sensitizer which displays more intense absorption than porphyrin in the red region: pheophorbide *a* exhibits a λ_{max} of 666nm versus 635nm for ALA-induced protoporphyrin IX[11-12]. Previous studies have demonstrated the therapeutic potential of pheophorbide *a*-based PDT on leukemia, colon cancer, hepatoma, and uterine carcinosarcoma[13-16]. Unfortunately, Hajri *et al.* have found that liposomal pheophorbide *a* at a dose of 30mg/kg led to much higher pheophorbide *a* levels in colon and gut than in HT29 tumor[14].

A previous study by this group predicted that the 1-hydroxyethyl derivatives of protoporphyrin IX or pheophorbide *a* showed a lower toxic potency than those of the parent compounds[26]. The 1-hydroxyethyl substituent increases the hydrophilicity of the compounds, which is an advantage when the drug is administered systemically, therefore it could impair uptake by cellular membranes, and consequently reduce toxicity. Furthermore, the 1-hydroxyethyl derivativess of protoporphyrin IX or pheophorbide *a* are found to generate oxygen more efficiently than those of the parent compounds when irradiated with visible light (data not shown). The 1-hydroxyethyl derivatives of protoporphyrin IX was synthesized using an addition reaction with hydrobromide, followed by nucleofilic substitution with H_2O[17].

In this work, the potential of protoporphyrin IX, pheophorbide *a* and the 1-hydroxyethyl derivatives for PDT of human epithelial cervix carcinoma (HeLa) and human breast cancer (T47D) cells lines are studied. Analysis of the dark toxicity was evaluated against normal cells (Vero) in addition to the cancer cells. Moreover, MTT assay was used to determine the inhibitory effects of test compounds on cell growth *in vitro*[18].



(1) R=CHCH$_2$
(2) R=CHOHCH$_3$

(3) R=CHCH$_2$
(4) R=CHOHCH$_3$

Fig. 1: Chemical structure of protoporphyrin IX (1), pheophorbide *a* (3) and its 1-hydroxyethyl derivatives (2,4)

MATERIALS AND METHODS

Chemicals

The 1-hydroxyethyl derivativess of protoporphyrin IX and pheophorbide *a* were synthesized at the School of Pharmacy, Bandung Institute of Technology (Bandung, Indonesia). Pheophorbide *a* was isolated and synthesized from *Spirulina platensis*. Dulbecco's modified Eagle medium (DMEM), M199, fetal bovine serum (FBS), fungizone 0.5%, and penicillin-streptomycin were purchased from Gibco (Invitrogen, USA). Tripsin-EDTA 0.025% was obtained from Gibco (Invitrogen, Canada. Protoporphyrin IX, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS) and all other chemicals were obtained from Sigma-Aldrich.

Cell culture

HeLa human epithelial cervix carcinoma and T47D human breast cancer cells lines were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 0.5% fungizone. Vero normal cells were maintained in M199 medium supplemented as above. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and were sub-cultured every 3-4 days using 0.025% Trypsin–EDTA solution.

Dark toxicity

HeLa, T47D and Vero cells were seeded into 96-well plates (100µL/well) at densities of 10000cells/well and incubated for 24 hrs. Afterwards, cells were washed with phosphate buffered saline (PBS), and 100µL of medium containing PS at a given concentration and 0.5% DMSO was added to each well, with the exception of control wells. The cells were incubated for 24 hrs, and then washed with PBS. Cell viability was determined using the MTT assay.

Light-dependent toxicity

HeLa and T47D cells were seeded into 96-well plates (100µL/well) at densities of 10000cells/well, and were incubated 24 hrs. Afterwards, cells were washed with phosphate buffered saline (PBS), and 100µL of medium without FBS, containing PS at a given concentration, and 0.5% DMSO, was added to each well, with the exception of control wells. Subsequently, the cells were exposed to light (300-850nm, maximum 610nm, 5mW/cm^2) from three mercury ML lamps (Philips 160 watt, The Netherlands) for 15 min. Directly after light exposure, the cells were incubated for 24 hrs. Cell viability was determined using the MTT assay.

MTT assay

After being incubated for 24 hours, the medium was discarded and replaced with MTT-containing medium (0.5mg/mL) and incubated for 4 hrs at 37°C, with 5% CO_2. The reaction was stopped with 10% SDS in 0.1M HCl solution and was incubated overnight in a light protected chamber to dissolve the formazan salt. The absorbance was measured with an ELISA reader at 595nm. Cell viability was expressed as the percentage of viable treated cells relative to untreated control cells.

RESULTS AND DISCUSSION

The compounds **1-4** were used to test for *in vitro* photosensitizing activity on HeLa human epithelial cervix carcinoma and T47D human breast cancer cells lines. The dark toxicities of the compounds on HeLa, T47D and Vero cell lines were analyzed at the same time. Cell cultures with photosensitizers were irradiated under similar conditions. The dark toxicities were studied to estimate the long-term side effects of these drugs.

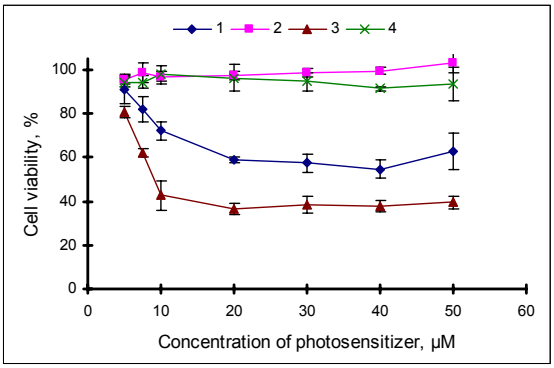


Fig. 2: Dark toxicity of 1-4 in normal cell (Vero). Error bars represent standard deviations (SD).

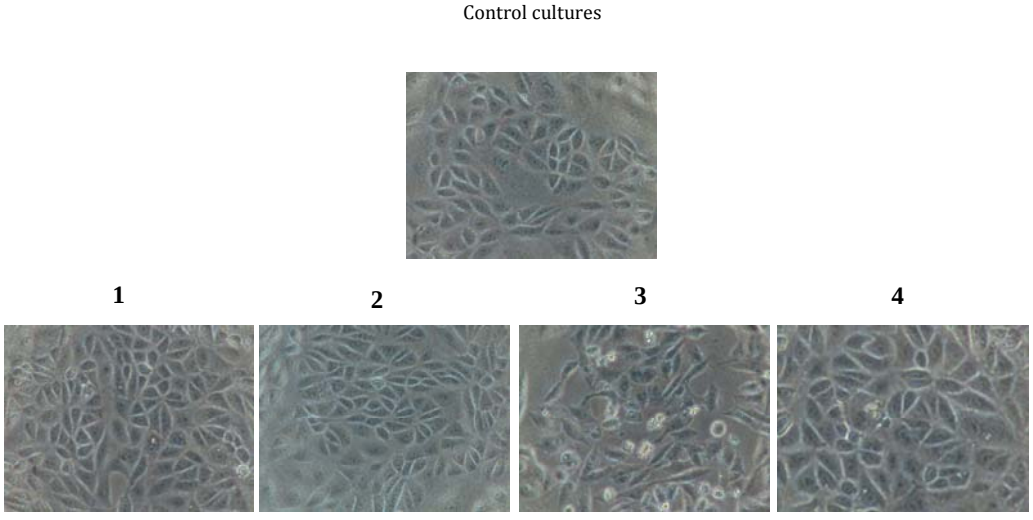


Fig. 3: Phase contrast images of Vero normal cells after incubation with 7.5µM of compounds 1-4 for 24 hours in dark condition

The dark toxicities of **1-4** in Vero cells are shown in Fig. 2. Protoporphyrin IX (**1**) and Pheophorbide *a* (**3**) had cytotoxic effects at a given concentration. Pheophorbide *a* exhibited the highest toxicity compared with that of all compounds studied. Protoporphyrin survival was 72.2%±4.1 for Vero cells, while Pheophorbide *a* was 42.7±6.7% at a concentration 10μM, and did not decrease further in the concentrations range from 20 to 50μM. Protoporphyrin IX tends to aggregate in aqueous solutions comparable with that of its 1-hydroxyethyl derivatives. This may play a role in moderating the level of cytotoxicity. Serious cytotoxicity and remarkable DNA damage was found in lymphocytes after ALA-induced protoporphyrin IX incubation as well as without light irradiation[9]. The chromosome aberrations and the induction of micronuclei were reported after ALA exposure to hepatocytes in the absence of light[19]. Furthermore, the degradation of cellular DNA was found after exposure of the isolated DNA to ALA[20].

The 1-hydroxyethyl derivativess of **1** and **3** had lower cytotoxic effects for Vero cells compared to those of the parent compounds. The cell survival was 95-100%. As previously seen, the 1-hydroxyethyl substituent increases the hydrophilicity of the compounds, which is an advantage when the drug is administered systemically, and could impair uptake by a cellular membrane, reducing toxicity as a result.

Similar results were obtained with HeLa cell lines. The 1-hydroxyethyl derivativess of **1** and **3** had lower cytotoxic effects when compared with those of the parent compounds (Fig 4). For compound **2**, no cytotoxicity was seen at concentrations up to 50μM. The previous study showed that compound **1** at concentrations from 0.8μg/ml to 20μg/ml was found to be cytotoxic to HeLa cells[21].

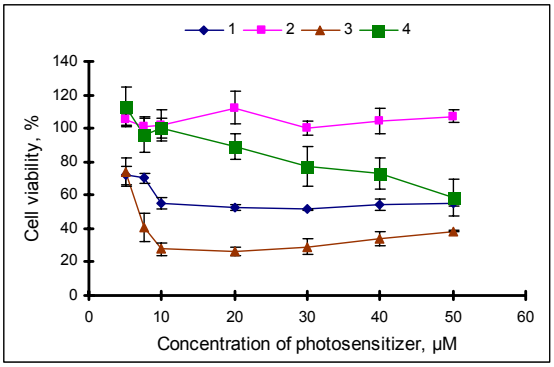


Fig. 4: Dark toxicity of 1-4 in HeLa cell lines. Error bars represent standard deviations (SD).

In the case of T47D cells, compound **2** is essentially non-cytotoxic at concentrations up to 50μM in the absence of light, but exhibits a high photocytotoxicity. In contrast, compounds **2** and **4** showed non-cytotoxic effects at concentrations up to 50μM and 30μM, respectively, compared to HeLa cells maintained in the dark under similar conditions. Studies published so far suggest that the mode of cell death induced by PDT is dependent on the sensitizer, the cell line used and the cell density[22-23]. In this study, different dark cytotoxicities were observed in different cell lines. This result indicates that the bystander effect may play a role[22-23].

Analysis of light-induced toxicity of compounds **1-4** in HeLa and T47D cells is shown in Fig. 5 and Fig. 6, respectively. The cells were treated with compounds **1-4** (5-50μM) and directly exposed to light. The corresponding LD_{50} values are summarized in Table 1, which shows that all of these compounds are highly potent. The cytotoxicity of photosensitizers **1-4** in the presence of irradiation is also stronger than that of those maintained in dark conditions. For compound **4**, the cell survival decreases to 6% at 20μM, while for compound **2** the cell survival decreases to 11% at 40μM in HeLa cell lines. The results for T47D cells were almost the same.

The phototoxicity of 1-hydroxyethyl derivativess was less than that observed for the parent compound. It is worth noting that although the 1-hydroxyethyl derivativess exhibit higher single oxygen quantum yields than their parent compounds in aqueous solutions (data not shown), the photocytotoxicity is lower than for the parent compounds. This is an indication that incubation time before exposure to light was short. The results are reflected by a decrease in cellular uptake of hydrophilic compounds **2** and **4**. As outlined by Kwitniewski *et al.*, phototoxicity was dependent on both the incubation time and light dose[23]. Moreover, the very sharp and intense Q band absorption spectra of PS in culture media should lead to a higher photosensitizing efficiency. Liu *et al.* reported the higher photocytotoxicity of the phtalocyanine compound, although the PS exhibits a lower single oxygen level than the other two analogues in DMF[24].

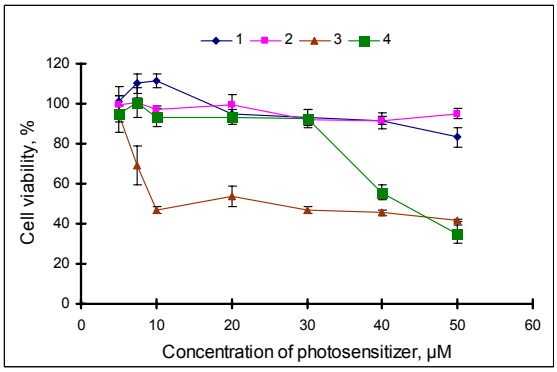


Fig. 5: Dark toxicity of 1-4 in T47D cell lines. Error bars represent standard deviations (SD).

Table 1: Photocytotoxicities of compounds 1-4 against HeLa and T47D cell lines

Compound	LC_{50} (μM)[a]	
	HeLa	T47D
1	7.0±0.04714	5.1±0.35
2	26.3±0.6	24.2±0.29
3	<5μM	<5μM
4	13.7±0.2	15.2±0.45

[a]Drug concentration that causes a 50% reduction in cell viability relative to untreated cells in the MTT assay. Values were obtained directly from cell-killing curves. Data are the mean from three independent experiments ± SD

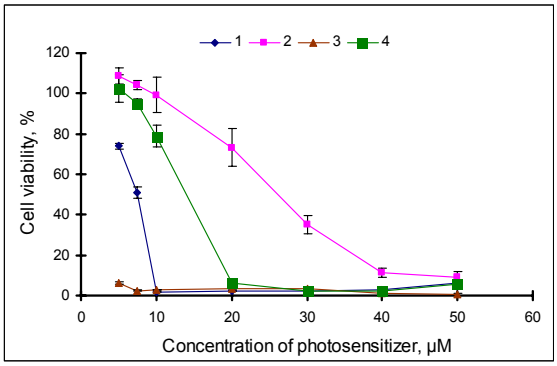


Fig. 6: Photocytotoxic profiles for HeLa cells exposed to 1-4 at different concentrations after photo-irradiation. For details, see the Expert. Part.

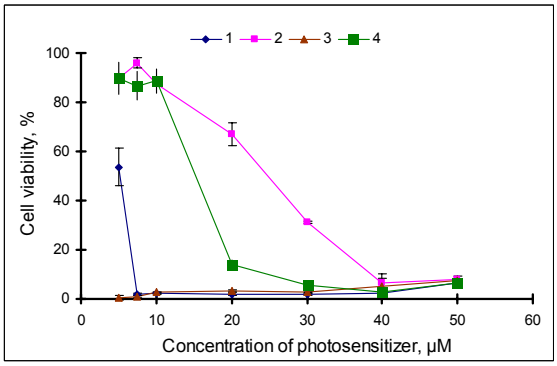
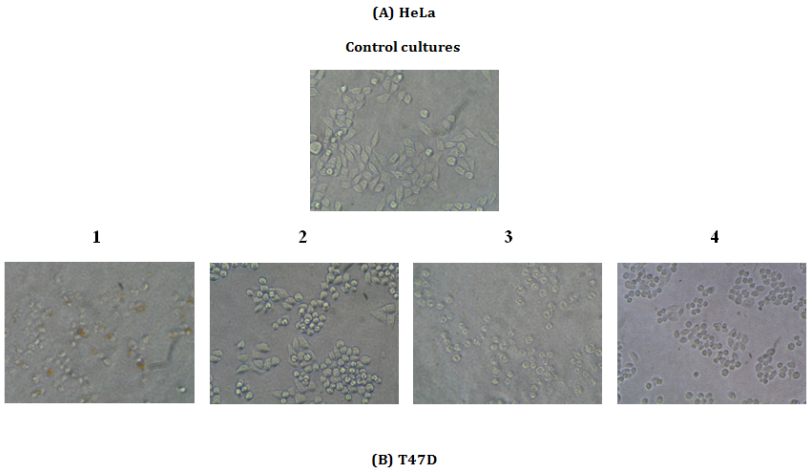


Fig. 7: Photocytotoxic profiles for T47D cells exposed to 1-4 at different concentrations after photo-irradiation. For details, see Expert. Part.

Cells treated with compounds **1-4** changed their morphology in comparison with non-treated cells (Fig 7), which is expressed in terms of their shapes. The population of cells undergoing apoptosis was observed in HeLa cells incubated with protoporphyrin IX[21]. The results for other compounds studied are similar. Changed morphologies, such as blebbing and cell shrinkage, were observed in treated cells, while non-treated cells retained their normal shape. Bednarz *et al.* revealed that protoporphyrin IX triggered chromatin condensation as well as fragmentation of nuclei in HeLa cells prior to PDT. However, the same alterations were also observed for other compounds studied in PDT[21].



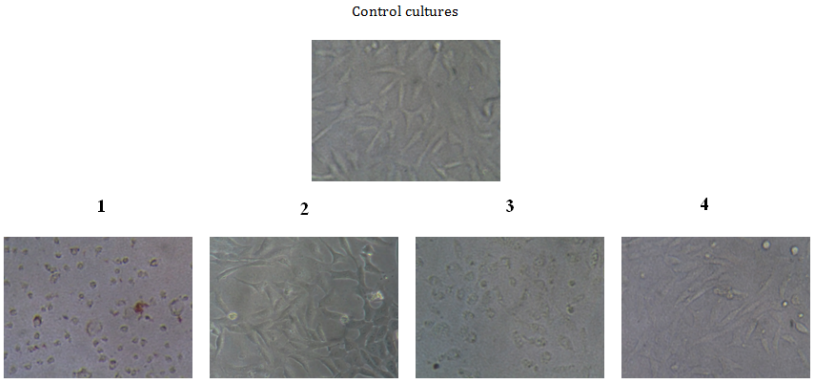


Fig. 8: Phase contrast images of (A) HeLa human epithelial cervix carcinoma and (B) T47D human breast cancer cells lines after being added with compound 1-4 at concentration 10µM, followed by light exposure.

The results of this study show that pheophorbide *a* was the most effective drug in both cell lines, but its dark toxicity was the highest in Vero, HeLa and T47D cell lines. The 1-hydroxyethyl derivatives of pheophorbide *a* **4**, showed lower cytotoxicity and higher effectiveness in both cell lines when compared with the other investigated compounds. The chlorin compound **4**, has substantially greater extinction coefficient in the red spectrum than porphyrins **1** or **2**, which is also an advantage that can increase the light penetration depth[25].

CONCLUSIONS

Protoporphyrin IX, pheophorbide *a*, and its 1-hydroxyethyl derivatives have been shown to be highly potent as PS for PDT. Replacing the vinyl group of **1,3** with 1-hydroxyethyl group can reduce its dark toxicity.

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