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Preparation and in vitro biological evaluation of tetrapyrrole ethanolamide derivatives as potential anticancer agents

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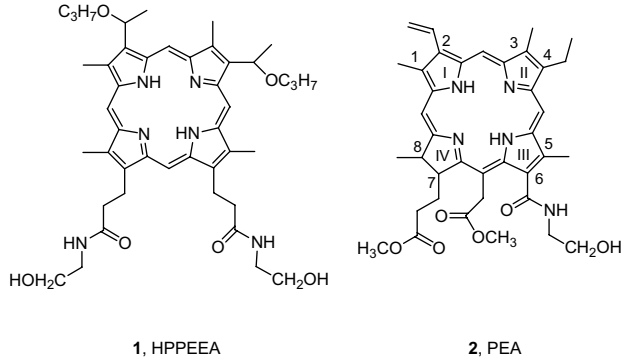
Abstract—Tetrapyrrole ethanolamide derivatives, **1** and **2**, were prepared from hematoporphyrin IX (HPIX, **3**) and methyl pheophorbide *a* (mPheo, **6**). These were evaluated for their dual action as chemotherapeutics and photosensitizers in treatment of cancer. The novel compounds showed significant in vitro anticancer activity as measured in different cell lines using the MTT assay and photodynamic activity measured by erythrocytes' photohemolysis.

The cyclic tetrapyrrole based dyes (e.g. porphyrins) have a potential use in medical applications, not only as a disease diagnostic tool, due to their fluorescence properties, but also as anticancer agents due to their photosensitization properties.[1] Photodynamic therapy (PTD) is a process that uses photosensitizer (PS) molecules that are capable of light absorption and generation of reactive oxygen species (ROS) such as singlet oxygen.[2] After administration of PS followed by irradiation with light the tumor cells are damaged. For examples, porphyrins, Photofrin®, Foscan®, and Visudyne® are clinically approved photosensitizers used in PTD to treat cancer and other diseases.[3]

The nitrogen mustards are a diverse class of compounds which share a common structural moiety and form a family of drugs useful in cancer therapy.[4] The principal antitumor reactivity of nitrogen mustards involves their interaction with DNA molecules.[5] A S_N2 type nucleophilic attack by the N-7 of the guanine base of DNA on the aziridinium ion intermediate (the active intermediate of N-mustard drugs) leads to the alkylation of DNA.[6,7] The result of the nitrogen mustard binding to the guanine is a defect in the DNA strand ultimately leading to cell death. A cross-link between two guanine bases on adjacent DNA strands can also occur if the nitrogen mustard contains two alkylating moieties. Rapidly proliferating cells, such as those found in neoplastic tissues, are the most sensitive to these DNA cross-linking agents, thus some selective tumor toxicity can be achieved.

Keeping all this in view, we have prepared two tetrapyrrole derivatives, hematoporphyrin propylether ethanolamide (HPPEEA, **1**) and pheophorbide *a* ethanolamide (PEA, **2**) (Fig. 1) from hematoporphyrin IX (HPIX, **3**) and methyl pheophorbide *a* (mPheo, **6**), respectively. The former derivatives were designed to incorporate an ethanolamide moiety, a known pharmacophore present in biologically active derivatives showing anticancer activity.[8] Moreover, the ethanolamide function was



1, HPPEEA **2**, PEA

Figure 1. Structure of hematoporphyrin propylether ethanolamide (HPPEEA, **1**) and pheophorbide *a* ethanolamide (PEA, **2**).

Keywords: Photodynamic therapy; Hematoporphyrin; Pheophorbide ethanolamide; Hematoporphyrin propylether ethanolamide.
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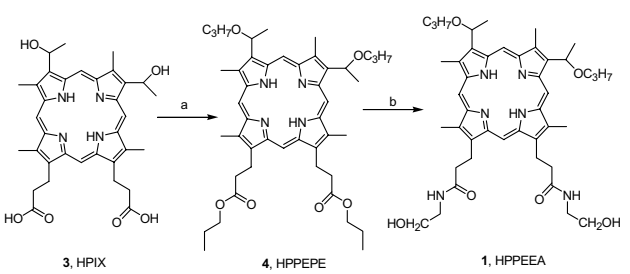
selected as it presents some structure similarities with the nitrogen mustards. Thus, compounds **1** and **2** could show interesting biological properties as dual action anticancer molecules. Consequently, these compounds were evaluated as chemotherapeutics as well as photosensitizers for the treatment of several types of cancers. The precursor derivatives, HPIX (**3**) and methyl pheophorbide *a* (mPheo, **6**), bearing no ethanolamide moiety were also tested on the same cancer cell lines in order to properly evaluate the real cytotoxic effects of the ethanolamide moiety on the final products, derivatives **1** and **2**.

Hematoporphyrin IX (**3**) was commercially available while methyl pheophorbide *a* (**6**) was obtained after isolation of chlorophyll *a* (**5**) from fresh spinach. The pheophorbide natural product was chosen because of its ease of preparation and its great capacity of singlet oxygen production (one of the most powerful ROS).

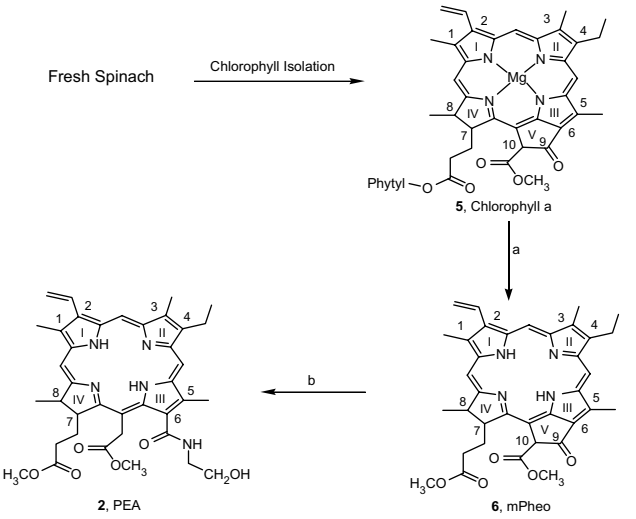
The synthesis of compound **1** (HPPEEA) was carried out starting from HPIX (**3**) (Scheme 1). Alkylation of **3** was done under strong acidic condition which yielded compound **4** (hematoporphyrin propylether-propylester, HPPEPE) in good yield. Condensation of compound **4** with ethanolamine in dioxane at reflux gave compound **1** (HPPEEA) in 76% isolated yield.

The synthesis of compound **2** was started from methyl pheophorbide *a* (**6**) which was initially obtained from chlorophyll *a* (**5**) isolated from fresh spinach (Scheme 2).[13] For this, chlorophyll *a* (**5**) was stirred for 2 h with acidic methanol at room temperature under an inert atmosphere of nitrogen which yielded methyl pheophorbide *a* (**6**) in good yield. The condensation of methyl pheophorbide *a* (**6**) with ethanolamine in dioxane was achieved with stirring at room temperature under dry nitrogen that gave compound **2** (PEA) in 45% yield upon ring opening of cyclopentenone ring (ring V). This ring opening is known in the literature[9].

The various compounds were characterized by the use of IR and NMR spectroscopy. In addition, the purity of methyl pheophorbide *a* (**6**) and its ethanolamide derivative PEA (**2**) was further assessed by HPLC which showed, in both cases, a purity exceeding 95%. This level of purity is comparable to other commercially available porphyrin products (Porphyrin Products, Logan, Utah, USA) that we tested.



Scheme 1. Reagents and conditions: (a) dry propanol, H^+, heat; (b) 2-aminoethanol, dioxane, reflux.



Scheme 2. Reagents and conditions: (a) H^+, CH_3OH, 21 °C; (b) 2-aminoethanol, dioxane, 21 °C.

The cytotoxic activity of the compounds was evaluated using the MTT cell proliferation assay.[10–12] The MTT assay was performed over an incubation period of 96 h under deem light to avoid decomposition. Different human neoplastic cell lines were tested to evaluate the chemotherapeutic activity of the ethanolamide derivatives HPPEEA (**1**), PEA (**2**) and their parent compounds, HPIX (**3**) and mPheo (**6**). Adriamycin, a chemotherapeutic drug of known activity, was used as a positive control and polyhematoporphyrin (pHP) which is a noncytotoxic PDT drug was used as a negative control. Table 1 gives the cell survival of various neoplastic cell lines at various drug concentrations. These are expressed as a percentage of untreated control cells (blank) which indicates that 100% survival indicates a noncytotoxicity situation.

For Adriamycin, the positive control, cytotoxicity starts at 0.01 μM in some of the cells and at 100 μM the survival in all cell lines was near 0%. pHP, the negative control, showed some cytotoxicity in the cell lines at about 50 μM and at the highest concentration (100 μM) the maximum response was about 20–60% cell survival. The drug concentrations required to cause a 50% survival (IC_{50}) are presented in Figure 2. These results confirm that Adriamycin is highly toxic, while pHP is essentially noncytotoxic.

HPIX (**3**) exhibited very little cytotoxic activity (Table 1 and IC_{50} in Fig. 2). However, at the highest concentration tested (100 μM) HPIX showed a little cytotoxicity. For example, the cell line K562 showed 30% survival. HPPEEA (**1**) exhibited significant cytotoxic activity (Table 1 and IC_{50} in Fig. 2). When 20 μM of HPPEEA was added to the cell lines, the survival dropped to near 10%. Below 20 μM, very little cytotoxicity is observed (Fig. 2).

Methyl pheophorbide *a* (mPheo, **6**) showed very little cytotoxicity (Table 1 and IC_{50} in Fig. 3). At the highest concentration of mPheo (100 μM) tested, the cell survival was near 80%. PEA (**2**) exhibited significant

Table 1. Percent cell survival of various neoplastic cell lines at various drug concentrations

Concentration (μM)	SIHA	K562	RAJI	HeLa	H69	H209	2780.9S	MDA.MB
Adriamycin								
0.01	ND	115.5 ± 23.9	110.0 ± 23.9	97.5 ± 9.7	86.5 ± 13.5	85.6 ± 14.0	107.6 ± 14.2	121.3 ± 16.9
0.1	72.2 ± 16.3	100.1 ± 25.8	105.6 ± 14.3	81.4 ± 13.3	82.2 ± 16.7	85.6 ± 17.1	105.4 ± 12.0	96.8 ± 12.9
10	4.4 ± 2.8	22.1 ± 8.5	8.6 ± 6.0	20.2 ± 24.2	37.71 ± 23.2	23.1 ± 18.7	56.9 ± 24.5	50.48 ± 29.2
50	3.5 ± 1.9	9.5 ± 6.3	2.5 ± 2.9	7.0 ± 4.7	16.8 ± 16.4	5.8 ± 2.9	13.8 ± 16.8	11.8 ± 10.2
100	ND	7.5 ± 0.0	1.1 ± 0.0	6.1 ± 0.0	6.6 ± 0.0	1.5 ± 0.0	7.4 ± 0.0	5.0 ± 0.0
Polyhematoporphyrin (pHP)								
0.1	ND	99.1 ± 0.0	91.5 ± 0.0	93.9 ± 0.0	95.7 ± 0.0	91.2 ± 0.0	88.9 ± 0.0	97.0 ± 0.0
1	97.1 ± 13.0	94.9 ± 9.0	103.9 ± 11.8	87.3 ± 5.8	84.9 ± 7.8	95.7 ± 8.9	92.8 ± 8.8	95.4 ± 0.2
10	97.0 ± 11.7	100.2 ± 3.0	102.4 ± 0.7	82.6 ± 24.7	81.4 ± 7.7	101.0 ± 11.8	101.0 ± 11.7	103.5 ± 5.8
50	89.9 ± 16.7	58.4 ± 17.1	54.2 ± 3.4	52.6 ± 25.8	70.0 ± 1.1	65.7 ± 0.0	60.0 ± 0.5	92.5 ± 22.5
100	69.4 ± 34.4	14.3 ± 0.0	17.7 ± 8.4	15.6 ± 7.6	29.0 ± 13.7	53.7 ± 26.6	18.1 ± 1.0	44.2 ± 10.6
Hematoporphyrin (*HPIX*, **3**)								
0.1	ND	68.7 ± 0.0	92.1 ± 0.0	85.8 ± 0.0	92.2 ± 0.0	91.4 ± 0.0	102.4 ± 0.0	135.7 ± 0.0
1	92.2 ± 5.2	82.7 ± 13.3	89.6 ± 15.9	102.6 ± 24.3	104.8 ± 16.4	94.0 ± 14.7	82.5 ± 15.3	115.4 ± 50.5
10	81.6 ± 9.3	82.5 ± 9.1	71.4 ± 24.1	113.0 ± 25.6	104.1 ± 12.3	99.5 ± 13.9	75.5 ± 28.8	103.8 ± 27.2
50	52.6 ± 7.7	55.9 ± 13.4	27.6 ± 14.7	109.1 ± 5.9	82.9 ± 6.2	93.3 ± 3.3	81.4 ± 22.7	107.3 ± 34.0
100	44.5 ± 18.9	32.1 ± 23.4	17.0 ± 4.6	104.4 ± 35.0	62.6 ± 1.3	84.6 ± 1.2	43.8 ± 37.4	82.8 ± 0.0
Hematoporphyrin propylether ethanolamide (*HPPEEA*, **1**)								
0.1	ND	72.9 ± 0.0	96.1 ± 0.0	99.6 ± 0.0	95.5 ± 0.0	97.8 ± 0.0	106.4 ± 0.0	137.4 ± 0.0
1	100.4 ± 13.4	84.7 ± 9.2	99.5 ± 7.0	118.7 ± 22.9	97.0 ± 19.8	104.9 ± 5.2	100.2 ± 0.2	112.0 ± 33.1
10	81.6 ± 9.3	82.5 ± 9.1	71.4 ± 24.1	113.0 ± 25.6	104.1 ± 12.3	99.5 ± 13.9	75.5 ± 28.8	103.8 ± 27.2
15.7	ND	28.5 ± 0.0	23.6 ± 0.0	7.8 ± 0.0	17.1 ± 0.0	16.1 ± 0.0	19.3 ± 0.0	24.3 ± 0.0
50	2.2 ± 1.4	5.7 ± 0.5	5.9 ± 3.0	7.4 ± 2.5	16.1 ± 1.5	8.6 ± 5.1	12.3 ± 4.0	7.4 ± 4.7
100	6.7 ± 3.5	6.67 ± 0.7	5.4 ± 0.9	10.5 ± 3.5	14.4 ± 1.3	9.8 ± 5.9	6.5 ± 1.60	5.2 ± 0.1
Methyl pheophorbide a (*mPheo*, **6**)								
0.1	ND	86.0 ± 0.0	92.9 ± 0.0	89.1 ± 0.0	70.7 ± 0.0	83.6 ± 0.0	100.0 ± 0.0	128.2 ± 0.0
1	98.0 ± 5.4	93.3 ± 1.4	101.8 ± 17.9	77.4 ± 8.5	84.3 ± 2.6	90.2 ± 8.4	94.9 ± 4.3	115.4 ± 31.1
10	90.8 ± 11.1	97.3 ± 3.1	106.7 ± 7.6	72.1 ± 3.6	79.0 ± 8.6	91.7 ± 2.1	93.4 ± 6.2	103.3 ± 16.1
50	81.0 ± 10.0	97.3 ± 0.0	106.4 ± 7.8	48.7 ± 26.7	76.7 ± 12.2	85.2 ± 1.8	90.1 ± 19.9	101.3 ± 15.8
100	68.0 ± 8.1	105.7 ± 15.0	94.3 ± 1.1	36.7 ± 34.1	63.6 ± 12.9	82.3 ± 3.7	83.2 ± 10.4	68.9 ± 0.00
Pheophorbide a ethanolamide (*PEA*, **2**)								
0.1	ND	129.4 ± 0.0	117.9 ± 0.0	126.0 ± 0.0	95.7 ± 0.0	101.9 ± 0.0	98.8 ± 0.0	109.8 ± 0.0
1	97.8 ± 5.6	112.6 ± 24.8	119.0 ± 8.0	113.6 ± 57.0	101.3 ± 10.5	102.0 ± 10.2	117.0 ± 7.1	120.7 ± 16.8
10	99.9 ± 8.6	115.2 ± 28.9	120.6 ± 0.1	112.6 ± 51.7	98.4 ± 14.8	106.0 ± 1.0	99.0 ± 9.7	107.4 ± 13.0
50	1.2 ± 1.0	6.1 ± 3.4	2.5 ± 3.1	6.3 ± 4.1	58.7 ± 7.9	59.4 ± 25.5	1.7 ± 1.1	1.6 ± 0.9
100	1.1 ± 0.7	2.9 ± 3.0	3.3 ± 4.5	7.8 ± 8.9	14.4 ± 12.3	5.2 ± 4.2	1.7 ± 0.4	1.6 ± 0.4

ND, not determined.

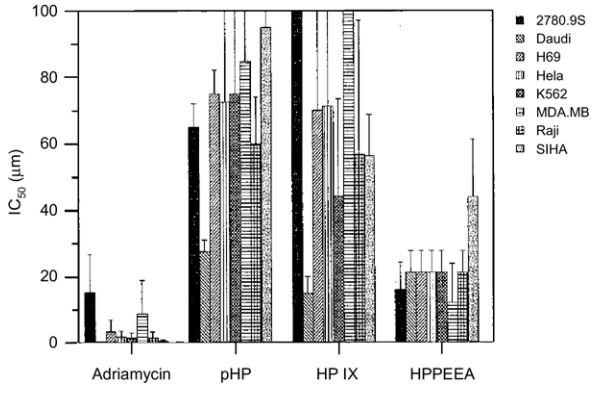


Figure 2. IC_{50} of HPIX (**3**) and HPPEEA (**1**) on several human neoplastic cell lines. Adriamycin and pHP are positive and negative controls, respectively.

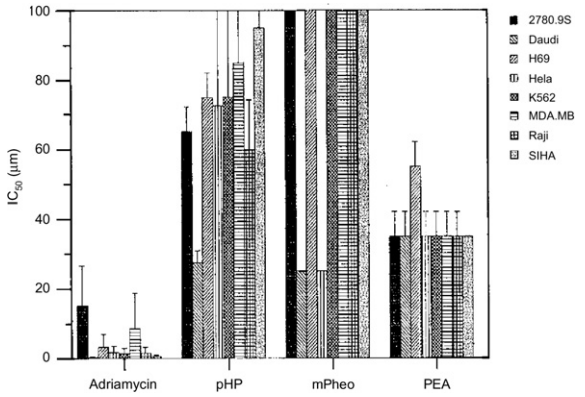


Figure 3. IC_{50} mPheo (**6**) and PEA (**2**) on several human neoplastic cell lines. Adriamycin and pHP are positive and negative controls, respectively.

cytotoxic activity (Table 1 and Fig. 3). For a 40 μM concentration PEA (**2**) showed less than 10% cell survival. This is similar to that of HPPEEA (**1**) but at twice its concentration.

Table 2. Inhibitory concentrations[a] of tetrapyrrole ethanolamide derivatives (**1** and **2**), precursors, and controls on various cancer lines

Cell lines	Adriamycin	pHP	HPIX (**3**)	HPPEEA (**1**)	mPheo (**6**)	PEA (**2**)
2780.9S	15 ± 1	65 ± 7	>100	16 ± 8	>100	35 ± 7
Daudi	0.3 ± 0.2	28 ± 4	15 ± 5	22 ± 6	25	35 ± 7
H69	3 ± 3	75 ± 7	70 ± 52	22 ± 6	>100	55 ± 7
HeLa	2 ± 1	73 ± 39	71 ± 50	21 ± 6	25	35 ± 7
K562	1 ± 1	75 ± 35	44 ± 29	22 ± 6	>100	35 ± 7
MDA.MB	9 ± 1	85 ± 21	>100	12 ± 12	>100	35 ± 7
Raji	2 ± 1	60 ± 14	57 ± 40	21 ± 6	>100	35 ± 7
SIHA	0.5 ± 0.3	95 ± 6	56 ± 12	44 ± 18	>100	35

[a] Concentrations (in μM) derived from MTT cytotoxicity assay required to obtain an IC_{50}.

The cytotoxic activity of both ethanolamide compounds (**1** and **2**) occurred over a very low concentration range. They had no effect below 10 μM but in the range 20–40 μM they caused almost 100% deaths. The activity of Adriamycin occurred in a much wider range (Figs. 2 and 3).

Table 2 and Figures 2 and 3 compare the response of different cell lines to the various drugs tested. From these it is apparent that the ethanolamides exhibit some cytotoxic activity but at higher concentration than that of Adriamycin. Also from these we find that PEA (**1**) requires about twice the concentration of HPPEEA (**2**) to accomplish 50% cell kill in some of the cell lines. The parent compounds of the ethanolamides, mPheo (**6**) and HPIX (**3**), had very little cytotoxic activity.

To further analyze the potential dual action of the novel compounds, the photosensitization activity of the compounds was evaluated by their photohemolytic activity. In the photohemolysis assay the amount of hemoglobin released from damaged red blood cells as a result of photosensitization is measured. The hemolysis results expressed as percentage of drug concentration are presented in Figures 4–7. The no light control in each test indicates that the drug alone had no effect on the hemolysis of the red blood cells, with the exception of PEA (**2**) where a small increase is observed (Fig. 7). In this case, at the maximum concentration used (250 nM) the per-

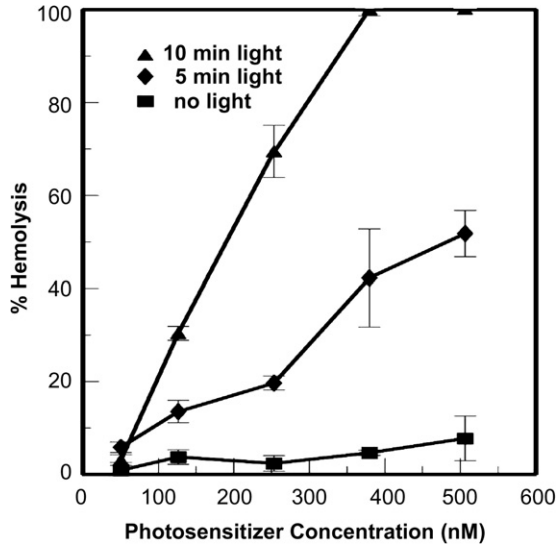


Figure 4. Photohemolysis measured at 414 nm for HPIX (**3**) treated red blood cells at different drug concentrations and light exposures.

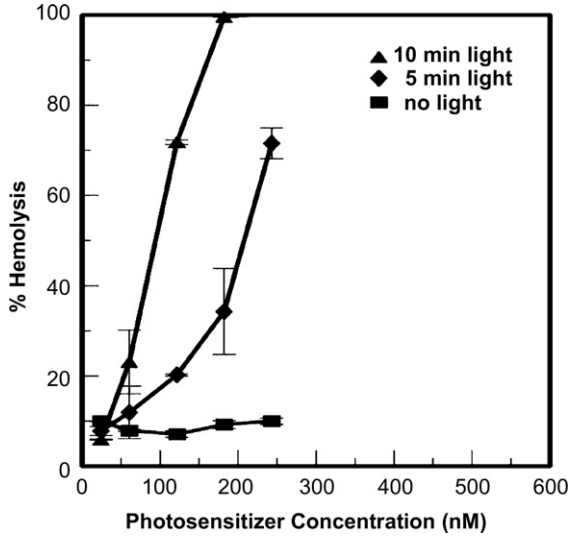


Figure 5. Photohemolysis measure at 414 nm of HPPEEA (**1**) treated red blood cells with different drug concentrations and light exposures.

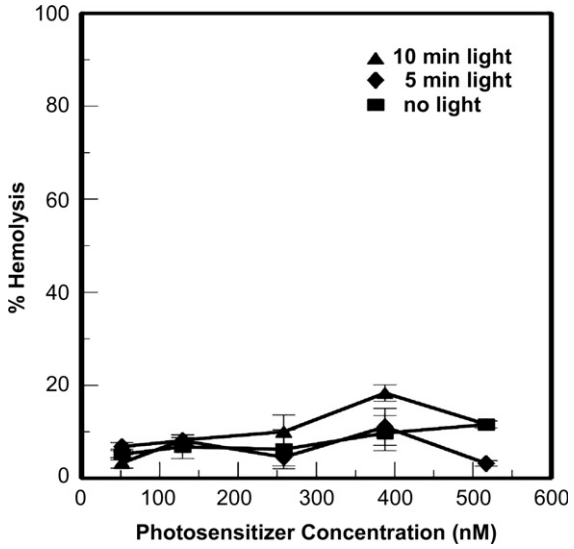


Figure 6. Photohemolysis of red blood cells measured at 414 nm treated with mPheo *a* (**6**) at different drug concentrations and light exposures.

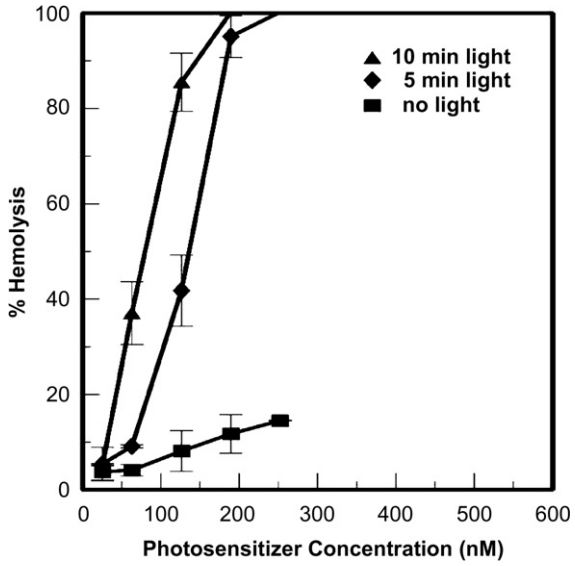


Figure 7. Photohemolysis of red blood cells measured at 414 nm treated with PEA (**2**) with different drug concentrations and light exposures.

cent hemolysis is 17%. This value is slightly higher than the control which is at the 0% level.

Figure 4 illustrates the photohemolysis of red blood cells by HPIX (**3**). At a concentration of about 375 nM with a 10 min light exposure it produced 100% hemolysis.

The photohemolysis of HPPEEA (**1**) is shown in Figure 5. A concentration of 125 nM with 10 min light exposure produced 100% photohemolysis. This is a threefold increase over that of HPIX (**3**).

Methyl pheophorbide *a* (**6**) had very little photohemolytic activity (Fig. 6). At the highest mPheo (**6**) concentration (520 nM), only 18% photohemolysis occurred. This is at background level.

Figure 7 shows the photohemolysis activity of PEA (**2**). A concentration of about 190 nM, with a 10 min light exposure, caused 100% hemolysis (Fig. 7). The concentration of PEA (**2**) that produced 100% hemolysis with 5 min of light was only slightly higher. From this we determine that PEA (**2**) has a high photohemolytic activity.

The summary of the photohemolysis data is given in Table 3 in which the drug concentrations required to produce 50% hemolysis are tabulated. PEA (**2**) exhibited the highest photohemolytic activity, followed by HPPEEA (**1**), HPIX (**3**) and lastly mPheo (**6**). While PEA (**2**) showed slightly higher activity after 5 min light exposure than HPPEEA (**1**), these ethanolamides have higher activity than their respective parent compounds mPheo (**6**) and HPIX (**3**), respectively.

Table 3. Summary of photosensitizer amount required for 50% hemolysis

Compound	5 min light exposure concentration (nM)	10 min light exposure concentration (nM)	Figure
HPPEEA (**1**)	90	210	5
PEA (**2**)	80	140	7
HPIX (**3**)	170	460	4
mPheo (**6**)[a]	>525	>525	6

[a] Only a small amount of photohemolysis was observed.

In conclusion, the compounds HPPEEA (**1**) and PEA (**2**) showed significant photosensitization activity as determined by photohemolysis as well as cytotoxic activity in different cell lines. The photohemolytic studies indicate that these ethanolamide derivatives showed good photosensitization activity. Furthermore, the MTT cytotoxicity studies of the compounds indicate that the derivatives **1** and **2** have significant chemotherapeutic activity. The ease of access of these novel compounds (**1** and **2**) and their promising biological properties may provide valuable tools in PDT for the treatment of diverse diseases. Further investigations of these ethanolamide derivatives are carried out to determine which compounds could be used as dual action anticancer agents presenting both photodynamic action as well as chemotherapeutic action for the treatment of various cancers. The therapeutic potential of the novel molecules will be further assessed in an animal model in our laboratory.

Acknowledgment

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13. *Chlorophyll extraction from spinach:* Chlorophyll *a* was extracted from locally purchased spinach leaves by solvent extraction. The plants' leaves were washed with water and separated into 100 g (fresh weight) lots. Methanol (500 mL), 0.5 g $NaHCO_3$, and 100 g spinach were combined in a 1 L flask. The leaf tissue was homogenated into a pulp using a tissue homogenator (Model L-04719-00 Cole Parmer). Room temperature was maintained during the grinding. The methanol was separated from the pulp by gravity filtration through fluted Whatman #1 filter paper. All methanol lots were pooled, and the extracted dye was partitioned into petroleum ether (35–60 °C grade), by adding equal parts of petroleum ether and water. The water–methanol partition was discarded, and the petroleum ether fraction was further washed with a 50 mM NaCl–H_2O solution. The resulting crude pigment extract was dried by rotary evaporation.
The pigments in the crude extract were separated by liquid column chromatography on silica gel using 5% *n*propanol in petroleum ether as the mobile phase. The chlorophyll *a* fractions were dried by rotary evaporation and used to synthesize methyl pheophorbide *a* (**6**) as described herein.
Procedure for the synthesis of ethanolamide derivatives: PEA (2): Methyl pheophorbide *a* (**6**) (1 g) was added to dioxane (20 mL) and ethanolamine (10 mL). The reaction mixture was stirred under nitrogen at room temperature but was refluxed in the case of HPPEEA (**1**). The progress of the reaction was monitored by tests on silica TLC plates using 5% methanol in dichloromethane as the mobile phase. The conversion reaction of mPheo (**6**) to PEA (**2**) took about 2 h. On completion of the reaction, the mixture was extracted into chloroform by adding equal parts of chloroform and water. The chloroform layer was washed several times with water and then neutralized by dropwise addition of concentrated HCl. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified by silica column chromatography using 5% methanol–chloroform. PEA (**2**) eluted from the column was dried and then placed in a Fischer pistol for 7 h at 80 °C. PEA was stored in vials purged with nitrogen at −3 °C. Yield: 54%; IR (cm^{-1}): 3307, 1735, 1655, 1602; ^1H NMR (δ): 9.62 (s, 1H, α-H), 9.60 (s, 1H, β-H), 8.80 (s, 1H, δ-H), 8.10 (dd, J = 12.0 Hz and 19.0 Hz, 1H, 2a-H), 6.80 (t, J = 5 Hz, 1H, 6-NH), 6.35 and 6.15 (dd, J = 18.0 Hz and 13.0 Hz, 2H, 2b-H_2), 5.50 and 4.75 (dd, JJ = 19 Hz and 19 Hz, 2H, γa-H_2), 4.45 (d, J = 8.0 Hz, 1H, 8-H), 4.38 (d, J = 8.0 Hz, 1H, 7-H), 3.70 (s, 3H, γ-$COOCH_3$), 3.70 (q, 2H, 4a-H_2), 3.60 (s, 3H, 7-$COOCH_3$), 3.50, 3.50, 3.30 (s, 3H × 3, ring 1,3,5-CH_3), 2.75 (m, 2H, 7a-H and 7b-H), 2.20 (m, 2H, 7a′-H and 7b′-H), 1.60 (d, J = 9.0 Hz, 3H, 8 a-CH_3),1.20 (t, J = 7.0 Hz, 3H, 4b-CH_3), −1.80 (s, 2H, ring N–H); ^{13}C NMR (δ): 171 C-7c, 170 C-6a, 167 C-γb, 128 C-2a, 122 C-2b, 102.2 C-γ, 102 C-β, 99 C-α, 94 C-δ, 62 C-6c(CH_2OH), 52 C-γc, 51.5 C-7d, 51 C-7, 49.9 C-8, 43 C-6b(NH–CH_2), 38 C-γa, 23 C-8a, 20 C-4a, 19 C-4b, 13 C-1a, 11 C-3a; MS (*m*/*z*) 666 [M^+, Ref. 9b].
HPPEEA (1): A similar procedure was used for the preparation of this compound. However, the reaction was performed at reflux instead of at room temperature. Yield: 76%; mp 139–40 °C; IR (cm^{-1}): 3350, 3310, 2955, 2925, 2860, 1640, 1090, 1070, 840; ^1H NMR (δ): 10.66–9.98 (4 s, 4H, H-5, 10, 15, 20), 6.70 (m, 2H, NN amide), 6.08 (q, J = 6.5 Hz, 2H, $CH_3C(OPr)H$-3,8), 4.28 (t, J = 7.0 Hz, 4H, $COCH_2CH_2$-13,17), 3.65–3.51 (m, 16H, CH_3-2,7,12,18 and $CH_3CH_2CH_2O$), 3.00 (q, 4H, J = 7.0 Hz, $COCH_2CH_2$-13,17), 2.90 (m, 4H, $2NHCH_2CH_2OH$), 2.74 (m, 4H, $NHCH_2CH_2OH$), 2.22 (d, J = 6.5 Hz, 6H, $CH_3C(OPr)H$-3,8), 1.79 (sextuplet, J = 7.0 Hz, 4H, $CH_3CH_2CH_2O$), 0.93 (t, J = 7.0 Hz, 6H, $2CH_3CH_2CH_2O$), −3.90 (s, 2H, NH-21,23); Anal. Calcd for $C_{44}H_{60}N_6O_6$; C, 68.72; H, 7.86; N, 10.93. Found: C, 68.52; H, 8.03; N, 10.79.
Photosensitization activity measured by photohemolysis: Photohemolysis involves the measurement of hemoglobin released from damaged red blood cells by absorption spectroscopy. The red blood cell damage is a result of photosensitization by the drug. The red blood cells and photosensitization drug are combined in the presence of light for a specified light dose. After 24 h in the dark the amount of hemoglobin released from the damaged cells was measured. Human whole blood (0.75 mL) was washed three times in TBS (5 mM Tris and 150 mM NaCl, pH 7.4, unless otherwise stated) and added to 500 mL of TBS. This stock blood solution had an absorbance of about 1.5 at 414 nm when all hemoglobin was released from the cells. The blood stock solution was dispensed in 10 mL aliquots into 100 mm standard plastic Petri dishes. The drugs were dispensed in μL quantities into the Petri dishes containing the blood in the dark in order to give the required nM drug concentrations. The solvent (95% ethanol) used did not cause any hemolysis. After addition of the drugs, the Petri dishes were gently agitated. The drugs tested were: pHP; HPIX (**3**); HPPEEA (**1**); mPheo (**6**); and PEA(**2**).
The light source used for the light treatment is composed of a light table with a plexiglass top and with two 96″ VHO cool white fluorescent lamps, giving 9 mW cm^{-2} of white light to the samples. The Petri dishes were exposed to: (1) no light; (2) 5 min; or (3) 10 min of light and then removed and placed in the dark for 24 h. The samples were then collected and centrifuged at 2000 rpm at 5 °C after the 24-h dark period. The released hemoglobin from lysed cells found in the supernatant was measured by absorption at 414 nm (the peak of the hemoglobin Soret band). The percent hemolysis was calculated by comparison with a standard curve. The 100% hemolysis reference was generated by lysis of a 10 mL aliquot of the same washed red blood cells as used for the samples with distilled water. The hemoglobin absorption at 414 nm was not affected by the drug due to the low drug concentrations in the samples.
MTT cytotoxicity assay[10–12]*:* The MTT cytotoxicity assay involves the measurement of cell viability after treatment with cytotoxic agents by the colorimetric change of the MTT dye. The cells are placed in 96-well flat-bottomed tissue culture trays. The drug is added after 24 h at the stated concentrations (0.001–100 μM). The cells are grown in the presence of the cytotoxic drug for 4 days, and then the MTT dye is added. The dye is enzymatically converted to a colored formazan in metabolically active living cells. Those cells that have been damaged or killed by the cytotoxic agent will not produce the blue colored formazan dye, which is measured by light absorption at 570 nm using a Dynatech model MR600 plate reader. Each drug concentration was tested in quadruplicate in each experiment. The drugs tested were: Adriamycin (Sigma Chemical Co., St. Louis, Mo, USA), a chemotherapeutic agent; polyhematoporphyrin (pHP); hematoporphyrin IX (HPIX, **3**) (Porphyrin products, Logan, Utah, USA); HPPEEA (**1**); mPheo (**6**); and PEA (**2**). The cell lines used for the assay are: K562, human erythroleukemia; Raji, human burkitt lymphoma; Daudi, human burkitt lymphoma (used only for IC_{50} study); HeLa, human cervical carcinoma; H69, human small cell lung carcinoma; 2780.9s, human ovarian carcinoma; MDA.MB human breast adenocarcinoma; S1HA, human cervical squamous carcinoma.