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# Antitumor Agents. Part 209: Pheophorbide-*a* Derivatives as Photo-Independent Cytotoxic Agents†

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**Abstract**—A methanolic crude extract of the plant *Garuga pinnata* Roxb. (Burseraceae) showed promising cytotoxic activity against a panel of human tumor cell lines in vitro, including KB and its drug-resistant sublines (Ferguson et al. *Cancer Res*. 1988, **48**, 5956). Pheophorbide-*a* and-*b* methyl esters (**3**,**4**) were isolated as active principles with broad photo-dependent cytotoxic activities in the micromolar range. These findings prompted SAR studies of known and novel pheophorbide-*a* derivatives as photo-dependent and photo-independent cytotoxic agents. The results showed that zinc-protoporphyrin IX (**10**), zinc $13^2$(*R*)-hydroxypheophorbide-*a* methyl ester (**22**), and zinc chlorin-*e*6 trimethyl ester (**13**) possessed photo-independent cytotoxic activity. Compounds **13** and **22** were the most active cytotoxic agents of the series (mean $ED_{50}$ $4.6 \pm 1.0$ μM and $5.7 \pm 0.7$ μM, respectively) against KB cells incubated in the dark. 

## Introduction

Pheophorbide-*a* derivatives, such as pheophytin-*a* and pheophorbide-*a* methyl esters, inhibit replication of a hepatoma tissue culture (HTC) cell line following light irradiation.[1] These pheophorbide-*a* derivatives are structurally related to porphyrins and are of current interest as photosensitizers for use in photodynamic therapy (PDT), a modality developed to treat cancer with a combination of light and photosensitizers.[2] At present, only photofrin II is approved by the USFDA to treat esophageal and endobronchial non-small cell lung cancers, as well as certain types of early-stage lung cancer.[3,4] Photofrin II is a complex mixture of hematoporphyrin dimer, trimer, tetramer and pentamer, as well as dehydration products with both ester and ether linkages.[2] Thus, potent, less complex photosensitizers would be valuable for clinical evaluation and drug development.

Our preliminary work led to the discovery of pheophorbide-*a* and -*b* methyl esters from *Garuga pinnata* (Burseraceae) as cytotoxic agents. However, the activity was extremely variable, and reproducibility was ultimately traced to the need for short periods of photoirradiation (daily routine microscopic examination of cultures). In order to extend these findings and establish structure–activity relationships (SAR), a series of pheophorbide-*a* derivatives was prepared and evaluated for cytotoxic activities in vitro. When compared with commercially available porphyrin-based compounds, such as protoporphyrin IX (**9**) and chlorin-*e*6 (**11**), pheophorbide-*a* derivatives showed greater photo-dependent cytotoxic activities. Pheophorbide-based photosensitizers represent good leads for the treatment of cancer using photodynamic therapy, but their effectiveness and utility is dependent on light penetration to the tissue, which greatly restricts their clinical utility. However, in the present work, certain metal analogues of pheophorbide-*a* and chlorin-*e*6 were found to exhibit potent but essentially photo-independent cytotoxic activity. Therefore, such compounds also represent novel lead molecules with a broader potential for anticancer drug development.

## Results and Discussion

### Natural products isolation and purification

The structures of chlorophyll-*a* (**1**) extracted from spinach, pheophorbide-*a* and -*b* methyl esters extracted

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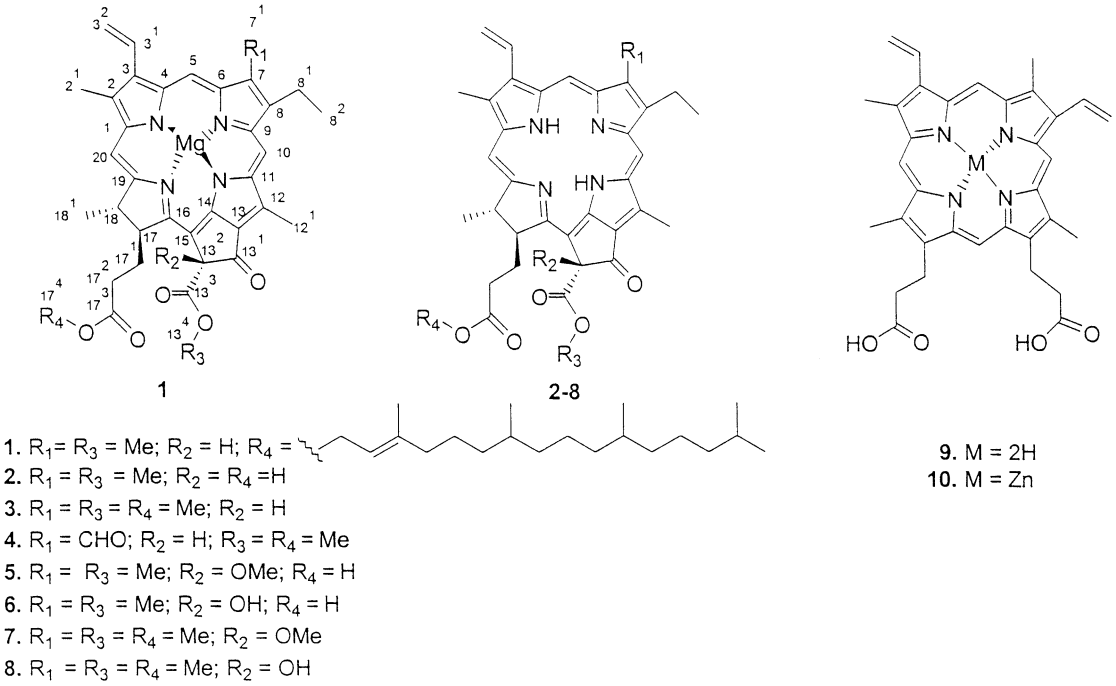
from *Garuga pinnata* Roxb. leaves (**3**,**4**), pheophorbide-*a* derivatives purified from *Clerodendrum calamitosum* Linn. leaves, and semi-synthetic derivatives (**2**, **3** and **5**–**8**) are shown in Scheme 1.[5]

## Synthesis

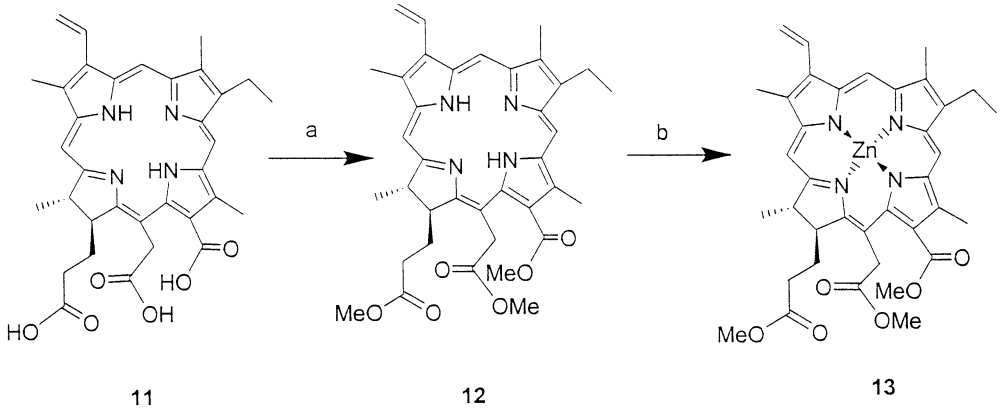
Syntheses of chlorin-*e*6 trimethyl ester (**12**) and the zinc derivative (**13**) of chlorin-*e*6 (**11**) are shown in Scheme 2. The synthetic Schemes affording pheophorbide-*a* derivatives are shown in scheme 3 (see Methods in Experimental). Pheophorbide-*a* (**2**), protoporphyrin IX (**9**), and chlorin-*e*6 (**11**) were purchased from porphyrin products (Logan, UT, USA). Zinc protoporphyrin IX (**10**) was purchased from Aldrich (Milwaukee, WI, USA).

Novel alkyl ester analogues of pheophorbide-*a* (**17, 19**–**21**) were obtained in 30–40% yield using a method described by Borovkov et al.[6] To prepare $13^2(R)$-hydroxy pheophorbide-*a* methyl ester, a hydrogen was abstracted from **3** at position $13^2$ using DBU, followed by hydroxylation with 10-camphorsulfonyloxaziridine as described in Dolphin et al.[7] (−)-(1*R*)-(10-Camphorsulfonyl)oxaziridine gave **14** selectively (>99% ee), while (−)-(1*S*)-(10-camphorsulfonyl)oxaziridine gave a mixture of **8** and **14** (55% ee, data not shown).

Because zinc protoporphyrin IX (**10**) possessed photo-independent cytotoxic activity (Tables 1 and 2), metal coupling pheophorbide derivatives were prepared. Metal chelation of free base analogues was highly dependent on both metal salt and solvent. Zinc(II) chloride did not generate zinc-coupled target compounds, but zinc(II) acetate provided adequate yields (20–30%). A non-polar sovent was preferable to a polar solvent, perhaps due to less ionic interaction between metal salt and solvent. Methanol was not a suitable solvent for this chelation, while refluxing in toluene-based solvent provided the desired products. With the same procedure, nickel pyropheophorbide-*a* methyl ester (**23**) was prepared from pyropheophorbide-*a*




**Scheme 1.** Structures of chlorophyll-*a* (**1**), pheophorbide derivatives (**2**–**8**), protoporphyrin IX (**9**) and zinc protoporphyrin IX (**10**).




**Scheme 2.** Synthesis of zinc chlorin-*e*6 trimethyl ester (**13**): (a) $CH_2N_2$; (b) $Zn(OAc)_2$, toluene, reflux, 2 h.

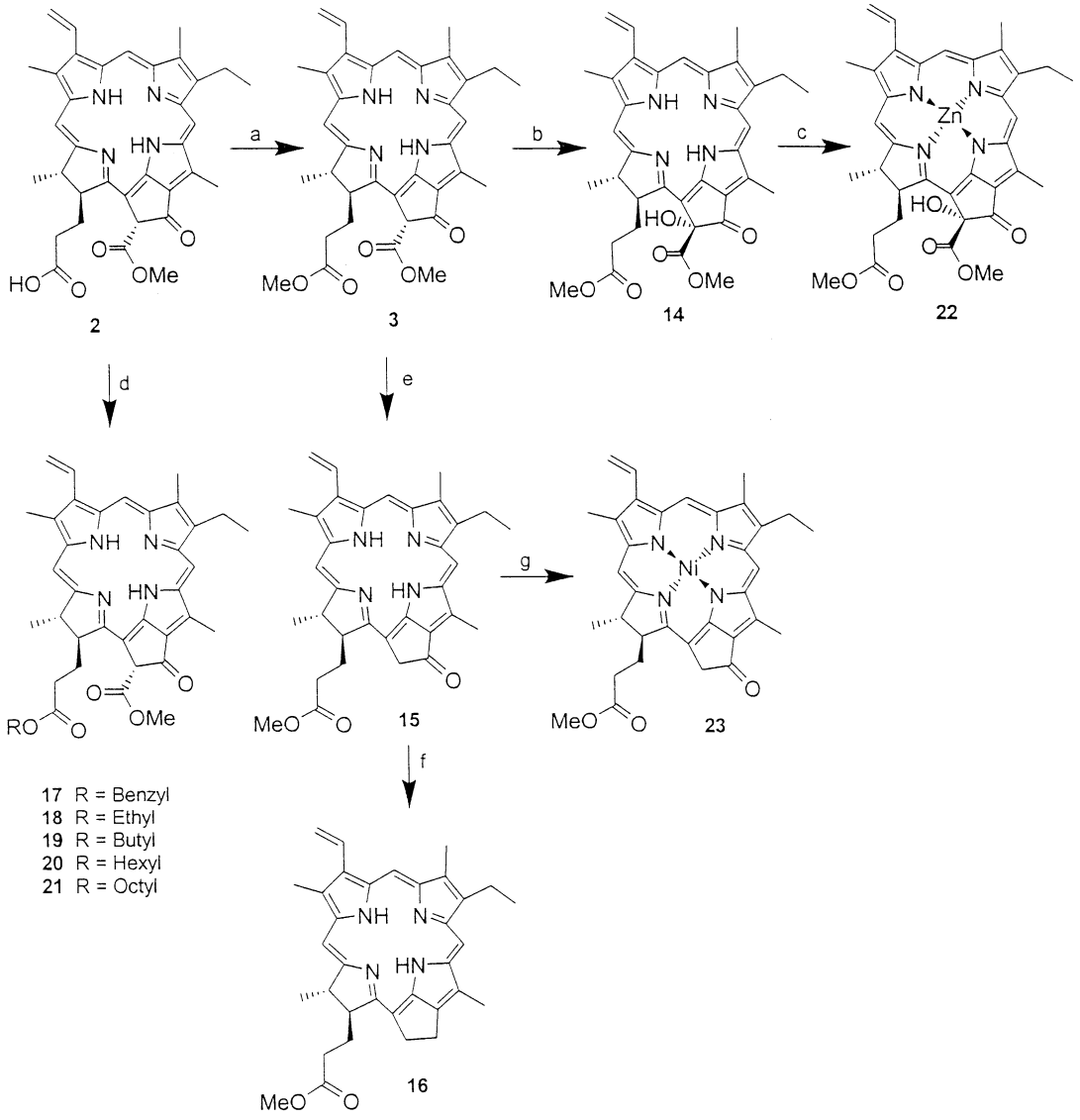
methyl ester (**15**) using nickel (II) acetate.

### In vitro evaluation

Nakatini et al. reported previously that pheophytin and its derivatives possessed photo-dependent cytotoxic activities.[1] We observed variable cytotoxic activity in our bioassay-guided fractionation and isolation study using *Garuga pinnata* Roxb. and further discovered that normal monitoring of the morphology of treated cells using light microscopy was sufficient to photo-activate the active principles, which were identified as pheophorbide-*a* and -*b* methyl esters (**3** and **4**, respectively). Therefore, in order to determine the SAR and photo-dependence of pheophorbide derivatives, it was important to compare cytotoxic activities between those using a standard photo-irradiation step and those determined in the dark. The photo-dependent cytotoxic activities of compounds **1**–**24** are shown in Table 1 and corresponding photo-independent activities (determined simultaneously using a dark condition for cell culture) are given in Table 2. 5, 10, 15, 20-Tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMPyP) (**24**, Scheme 4) was also evaluated using both treatment conditions and was found to be less cytotoxic than pheophorbide and chlorin derivatives (Tables 1 and 2). TMPyP interacts with DNA and, upon irradiation, causes DNA damage leading to cell death.[8] Because TMPyP was reported to produce singlet oxygen, the compound could directly induce DNA damage through electron transfer from guanine base to the bound photo-excited drug.[9] To determine whether pheophorbide derivatives act by causing DNA damage, DNA breaks were measured using a gel lysis assay.

### Gel lysis assay

The induction of double-stranded (ds) cellular DNA breaks by selected compounds was determined using a semi-quantitative gel lysis assay (Fig. 1). Interestingly,




**Scheme 3.** Synthesis of pheophorbide-*a* derivatives (**3**, **14**–**22**): (a) $CH_2N_2$; (b) DBU, −25 °C, followed by (−)-(1*R*)-(10-camphorsulfonyl)oxazirdine, −25 °C; (c) $Zn(OAc)_2$ or $Ni(OAc)_2$, toluene, reflux, 2 h; (d) ROH/$Boc_2$/DMAP, reflux, 1 h; (e) 2,4,6-collidine, reflux, 1.5 h; (f) $NaBH_4$, TFA, reflux, 3 h; (g) $Ni(OAc)_2$, toluene, reflux, 2 h.

among the compounds tested, **13** and **22** caused ds-DNA breaks under the dark treatment conditions. In contrast, **8**, **11** and **16** did not cause DNA breaks without a photo-irradiation step (Fig. 2). Overall results for compounds from photo-dependent and photo-independent groups are given in Table 3. On the basis of the results, DNA is a possible target of cytotoxic pheophorbide compounds. The mechanism of action of photo-independent compounds is currently under investigation and will be reported elsewhere.

**Table 1.** Photo-dependent cytotoxic activities[a] of pheophorbide-*a*, chlorin-*e*6 and porphyrin analogues against human tumor cell lines panel (KB, KB-VCR, KB-7d, SK-MEL-2), compared with human embryonic lung cell line (HEL)

| Compound | $ED_{50}$ (μM) | | | | |
|---|---|---|---|---|---|
| | KB | KB-VCR | KB-7d | SK-MEL-2 | HEL |
| **1** | 24.4±0.9 | 23.7±2.2 | 17.5±0.5 | 38.4±0.1 | 21.4±3.3 |
| **2** | 0.8±0.1 | 0.8±0.1 | 0.4±0.0 | 2.9±0.7 | 1.3±0.3 |
| **3** | 0.6±0.1 | 4.1±0.1 | 0.3±0.1 | 1.8±0.0 | 0.8±0.2 |
| **4** | 11.3±0.3 | >8 | >8 | 28.2±1.3 | >8 |
| **5** | 0.6±0.0 | 0.6±0.1 | 0.5±0.1 | 3.6±0.2 | 0.9±0.2 |
| **6** | 3.0±0.1 | 3.3±0.2 | 1.5±0.1 | >16 | 4.0±0.5 |
| **7** | 0.4±0.1 | 1.8±0.2 | 0.4±0.1 | 0.7±0.0 | 0.6±0.1 |
| **8** | 0.2±0.0 | 1.6±0.2 | 0.3±0.02 | 0.6±0.2 | 0.5±0.1 |
| **9** | >18 | 3.7±0.9 | 13.7±0.9 | 24.8±3.5 | 4.4±0.3 |
| **10** | 14.7±1.4 | 12.8±0.9 | 12.6±0.9 | 14.0±1.2 | 12.0±0.5 |
| **11** | 16.4±1.3 | 16.8±1.3 | 15.4±0.8 | 33.5±0.8 | ND[a] |
| **12** | 0.5±0.0 | 3.7±0.2 | 0.6±0.1 | 1.7±0.3 | 0.8±0.2 |
| **13** | 2.4±0.1 | 3.3±0.2 | 2.1±0.1 | 1.7±0.1 | ND[a] |
| **14** | 0.5±0.1 | 2.1±0.2 | 0.4±0.0 | 1.0±0.2 | 0.3±0.1 |
| **15** | 0.8±0.1 | 10.9±1.7 | 0.8±0.1 | 2.3±0.5 | 1.6±0.2 |
| **16** | 3.4±0.4 | 9.1±0.1 | 7.9±0.2 | 3.3±0.5 | >3 |
| **17** | 21.3±0.1 | 28.0±0.2 | >29 | 22.8±0.1 | 14.4±0.3 |
| **18** | >16 | >16 | >16 | 29.8±0.5 | >16 |
| **19** | 5.9±0.1 | >15 | 5.1±0.1 | 6.7±0.1 | 6.0±0.5 |
| **20** | 13.0±0.3 | >15 | 12.7±0.3 | 24.1±3.2 | 14.6±1.0 |
| **21** | 13.9±0.3 | >14 | 10.8±0.3 | 26.3±0.3 | >14 |
| **22** | 1.8±0.0 | 3.1±0.1 | 1.9±0.1 | 2.5±0.4 | 1.7±0.1 |
| **23** | 87.7±3.3 | 87.7±2.9 | 54.6±3.5 | ND[b] | ND[b] |
| **24** | >100 | >100 | >100 | >100 | >100 |
| **VP16** | 4.0±0.0 | >15 | ND[b] | ND[b] | ND[b] |

[a]Determined using a sulforhodamine B-staining assay of Rubinstein et al.[20] Cultured cells were observed at daily intervals using a Nikon ×−1 inverted microscope (40×magnification) irradiated by microscopic light for 2–3 min each day over 3 days before processing. Results are mean concentration (μM) of compounds that inhibit 50% growth. Standard deviation values were calculated from results of independent triplicate assays. VP-16 was included as a control.
[b]ND, not determined.

## Structure–activity relationships

The in vitro cytotoxicity assay results (Tables 1 and 2) led to the following SAR conclusions: (a) at C-7,[1] an aldehyde group (**4**) decreased cytotoxic activity at least 10–14-fold, compared with a methyl group (**3**); (b) at C-13[1], a carbonyl group was necessary for cytotoxic activity against KB cells (cf., **15** and **16**); (c) compounds with a carboxylic acid substituent at position 17[4] (**2**, **5**,

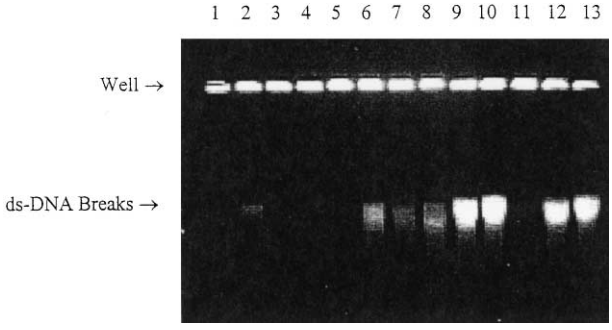



**Figure 1.** A representative result showing double-stranded (ds) cellular DNA breaks detected using gel lysis assay. (1) KB (dark); (2) VP16 (20 μM); (3) KB (dark); (4) KB (irr); (5) **2** (8 μM, dark); (6) **2** (4 μM, irr); (7) **5** (3 μM, irr); (8) **6** (15 μM, irr); (9) **3** (3 μM, irr); (10) **7** (2 μM, irr); (11) **8** (2 μM, dark); (12) **8** (1 μM, irr); (13) **14** (3 μM, irr).

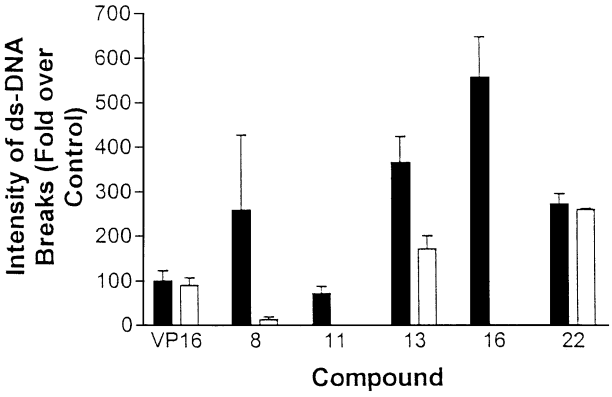



**Figure 2.** Double-stranded (ds) DNA breaks using gel lysis assay of photo-dependent cytotoxic agents (**8**, **11**, **16**) and photo-independent cytotoxic agents (**13**, **22**). Open bars represent compounds evaluated using the dark culture condition, and filled bars represent activities under a matched photo-irradiated condition. Compounds were tested as described in the experimental section and results are the mean ±SEM from two independent experiments.

**Table 2.** Cytotoxic activities of pheophorbide, chlorin and porphyrin analogues in the absence of light [a] against human tumor cell lines

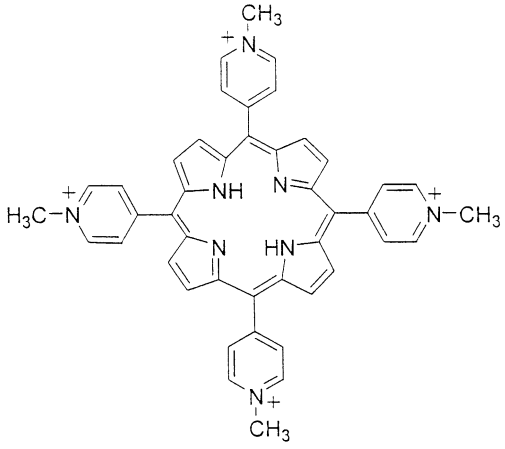
| Compound | $ED_{50}$ (μM) | | | | |
|---|---|---|---|---|---|
| | KB | KB-VCR | KB-7d | SK-MEL-2 | HEL |
| **1** | 21.6±1.0 | 26.1±1.1 | 18.7±1.2 | ND[b] | 12.8±0.0 |
| **10** | 17.9±0.8 | 19.6±1.4 | 15.6±1.9 | 15.8±1.8 | 10.4±1.6 |
| **13** | 4.6±1.0 | 5.1±1.3 | 5.0±0.7 | 4.7±0.7 | ND[b] |
| **22** | 5.7±0.7 | 5.8±0.7 | 5.1±0.1 | 6.1±0.4 | 4.4±0.7 |
| **23** | 69.5±3.3 | 69.5±2.5 | 56.3±2.1 | ND[b] | ND[b] |
| **24** | 41.1±1.6 | 34.5±1.3 | >50 | >50 | ND[b] |

[a]Determined using a sulforhodamine B-staining assay of Rubinstein et al.[12] Plates were covered by aluminum foil and incubated for 3 days and cultures were observed using a Nikon ×−1 inverted microscope after 3 days immediately before processing. Results are mean concentration (μM) of compounds that inhibit 50% growth. Standard deviation values were calculated from results of independent triplicate assays. Compounds **2**–**9**, **11**, **12**, and **14**–**21** were inactive at 40 μg/mL in the absence of light; therefore, they were excluded from this table.
[b]ND, not determined.

**Table 3.** Intracellular DNA damage [a] induced by pheophorbide, chlorin, and porphyrin derivatives

| Compound | Intensity of double stranded-DNA breaks (fold over control) | |
|---|---|---|
| | Irradiated | Dark |
| **2** | 96.8±14.0 | ND[b] |
| **3** | 256.0±147.0 | ND[b] |
| **4** | 85.3±47.7 | 4.1±1.1 |
| **5** | 90.1±88.7 | ND[b] |
| **6** | 94.4±5.20 | ND[b] |
| **7** | 687.0±201.0 | ND[b] |
| **8** | 259.0±168.0 | 12.6±6.3 |
| **10** | 341.0±68.0 | ND[b] |
| **11** | 72.1±16.0 | 0 |
| **12** | 238.0±48.5 | ND[b] |
| **13** | 366.0±58.0 | 171 |
| **14** | 444.0±107.2 | ND[b] |
| **15** | 142.0±45.4 | ND[b] |
| **16** | 558.0±147.0 | 0 |
| **19** | 241.0±90.1 | ND[b] |
| **21** | 68.9±15.0 | ND[b] |
| **22** | 272.0±74.6 | 259.2±2.8 |
| **VP-16** | 100.0±22.9 | 90.0±17.2 |

[a]Intracellular DNA breaks were studied using the gel-plug method as described in Bastow et al.[22] Standard error values, where indicated, were calculated from results of independent duplicate assays. The semi-quantitative values were the fold of increased intensity in DNA damage compared to control. The numeric values were determined with densitometry of ethidium bromide-stained agarose gels using STORM and ImageQuant 5.0 software. Weakly cytotoxic compounds were excluded from this assay ($ED_{50} > 10$ μg/mL). VP-16, a topoisomerase II inhibitor that induced double-stranded protein-linked DNA breaks, was included as a positive control in certain experiments.
[b]ND, not determined.



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**Scheme 4.** Structure of 5, 10, 15, 20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (**24**).

**6**) were not recognized as *p*-glycoprotein substrates; (d) at C-17[4], a methyl ester group (**3**) was preferred to other alkyl esters (**17**–**21**) for KB cytotoxicity; (e) replacing a hydrogen at C-13[2] (**3**) with a hydroxy (**8**) or a methoxy group (**7**) improved cytotoxic activity (2–3-fold) against KB cells; (f) the stereochemistry of the hydroxy group at C-13[2] of pheophorbide-*a* methyl ester was not an important determinant of cytotoxic activity in resistant cell lines (cf., **8** and **14**); (g) the free bases (**9**, **12**, **14**, **15**) possessed photo-dependent cytotoxic activity; (h) although metal chelated compounds (**10**, **13**, **22**, **23**) were 4–5-fold less cytotoxic than the corresponding free bases (**9**, **12**, **14**, **15**) in the presence of light, the action of the metal analogues was largely light-independent; (i) the metal chelated complexes (**10**, **23**) were poorer substrates of *p*-glycoprotein, except **13** and **22**; (j) the nickel analogue of pyropheophorbide-*a* methyl ester (**23**) was inactive both in the dark or in the presence of light.

## Conclusions

Generally, pheophorbide-*a* derivatives with longer alkyl esters at C-17[4] were only weakly cytotoxic compared to the methyl ester analogue (**3**); however, the butyl ester analogue (**19**) was more active than ethyl (**18**), hexyl (**20**), octyl (**21**), or benzyl (**17**) ester analogues under photo-irradiation (Table 1). The free-base analogues (**9**, **12**, **14**, **15**) were photo-dependent cytotoxic agents (no activity under the dark culture condition at 40 μg/mL), while the zinc-chelated analogues (**10**, **13**, **22**) displayed improved photo-independent cytotoxic activities in the micromolar range (Table 2). Interestingly, cytotoxic activities of zinc chelates compared to their free-base analogues (cf., **12** and **13**; **14** and **22**) decreased around 5–10-fold under photo-irradiation, but their activities increased more than 40-fold in the dark. In contrast, the nickel analogue (**23**) was inactive under either drug treatment condition.

Theoretically, singlet oxygen is the major cytotoxic species during photodynamic therapy, although evidence is lacking for its production in vivo. The photodynamic action of photosensitizers is initiated by photon absorption, which induces singlet oxygen production and subsequently causes DNA, RNA, or protein damage via oxidative stress. Molecular oxygen plays a key role in molecular damage, resulting in vascular collapse, tissue degradation, and cell death.[2] It has been shown that certain metal groups may affect singlet oxygen formation.[3] Therefore, nickel may also prevent such

production, while zinc may potentiate production, causing oxidative stress without requiring photo-irradiation. Although pheophorbide-*a* can generate singlet oxygen after photo-irradiation,[10] the metal chelated compounds may have other novel mechanisms of action, including DNA interaction leading to cellular ds-DNA breaks as reported herein. Additional mechanistic studies are needed to evaluate and further develop photo-independent pheophorbide-*a* derivatives as anticancer drug candidates.

## Experimental

### General experimental procedures

All melting points were determined on a Fisher–Johns melting point apparatus and are uncorrected. $^1$H NMR spectra were obtained using a Varian 300 MHz NMR spectrometer with TMS as the internal standard and $CDCl_3$ as solvent. All chemical shifts are reported in ppm. FAB-MS spectral analyses were determined on a VG Analytical VG-70E spectrometer, using chloroform as a solvent. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated aluminum silica gel sheets (Kieselgel 60 F-254). Preparative TLC was carried out on 1 mm Analtech precoated silica gel plates (Kieselgel 60-F254). The solvent systems used are described under syntheses of each compound. The UV–vis spectrum was obtained on a Beckman DU-600 UV–vis spectrophotometer, using chloroform as a solvent. For the cytotoxicity assay, a THERMOmax$^{TM}$ microplate reader (Molecular Devices Inc., Menlo Park, CA, USA) was used to measure the absorbance. Compounds were obtained as amorphous, green powders unless indicated otherwise.

**Chlorophyll-*a* (1). $C_{55}H_{72}N_4O_5Mg$**. Chlorophyll-*a* purchased from Aldrich was found to contain at least three major constituents (TLC in $CHCl_3$/hexane/EtOAc 1:3:1), therefore it was isolated from fresh spinach leaves to ensure sufficient pure compound for biological testing. Fresh leaves (1 kg) were dried at 60 °C overnight providing dried leaves (40 g) as starting material (4% w/w), which were macerated in 80% MeOH for 3 days. The extract was evaporated under vacuum providing 3.5 g of dark green residue (8.8% yield, dried weight basis). This crude extract was purified using repetitive silica column chromatography (2.5×25 cm), eluting sequentially with $CHCl_3$/hexane (4:1), $CHCl_3$/hexane/MeOH (9:1:0.1), and $CHCl_3$/MeOH (95:5), and MeOH by collecting 30-mL fractions. Chlorophyll-*a* was obtained by using a mixture of $CHCl_3$/hexane (4:1) as the eluent (5 mg; 0.012% yield from dried leaves). $^1$H NMR data were consistent with those in the literature;[11] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 412 (18,309); 536 (6368); 611 (2925); 668 (8418); FAB-MS: *m*/*z* 894 $(M+H)^+$.

**Pheophorbide-*a* (2).** Dark brown powder purchased from Porphyrin Products (Logan, UT, USA).

**Pheophorbide-*a* methyl ester or 14-ethyl-3-(2-methoxy-carbonyl-ethyl)-4, 8, 13, 18-tetramethyl-20-oxo-9-vinyl-23*H*, 25*H*-phorbine-21-carboxylic acid methyl ester (3). $C_{36}H_{38}N_4O_5$**. Compound **3** was obtained semi-synthetically and was also isolated as a natural product. For synthesis, a solution of pheophorbide-*a* [200 mg; Porphyrin Products (Logan, UT, USA)] in $Et_2O$ and MeOH (1:1; 50 mL) was treated with diazomethane at 0 °C with stirring for 15 min. The solvent was evaporated and the residue was purified by silica gel column chromatography, using $CHCl_3$/hexane/EtOAc (1:3:2) as the eluent. Recrystallization of **3** from $CH_2Cl_2$/MeOH provided the desired compound as dark blue crystals (190 mg; 95% yield). For phytochemical isolation, dried leaves (4 kg) of *Garuga pinnata* Roxb. were treated as above to give an 80% MeOH extract (330 g). This extract was partitioned between $H_2O$ and $CHCl_3$. The $CHCl_3$ extract was partitioned between hexane (10 g extract) and 90% MeOH (114 g extract). From the latter extract (114 g), **3** was purified by silica gel column chromatography, using $CHCl_3$/hexane/EtOAc (1:3:2) as the eluent, yielding 200 mg (0.005% yield from dried leaves). Mp 238 °C (lit.[12] 241 °C, lit.[13] 228 °C); $^1$H NMR[1,7,12] and UV–vis data[14] were consistent with literature values; FAB-MS: *m*/*z* 607 $(M+H)^+$.

**Pheophorbide-*b* methyl ester or 14-ethyl-13-formyl-3-(2-methoxy-carbonyl-ethyl)-4, 8, 18-tetramethyl-20-oxo-9-vinyl-23*H*, 25*H*-phorbine-21-carboxylic acid methyl ester (4).** $C_{36}H_{36}N_4O_6$. From a 90% methanolic extract of *Garuga pinnata* Roxb. leaves, **4** was purified by silica gel column chromatography, eluted with $CHCl_3$/hexane/EtOAc (1:3:2), yielding 66 mg of yellow powder (0.002% yield from dried leaves). Mp 218 °C (lit.[15] 270 °C); $^1$H NMR data were consistent with those in the literature;[1,16] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 439 (10,665); 528 (1434), 600 (1108), 655 (2728); FAB-MS: *m*/*z* 621$(M+H)^+$.

**13$^2$(*S*)-Methoxy pheophorbide-*a* (5); 13$^2$(*S*)-hydroxy pheophorbide-*a* (6); 13$^2$(*S*)-methoxy pheophorbide-*a* methyl ester (7); 13$^2$(*S*)-hydroxy pheophorbide-*a* methyl ester (8).** The method to isolate and modify these compounds is described in Cheng et al.[5]

**Protoporphyrin IX (9).** Red powder purchased from Porphyrin Products (Logan, UT, USA).

**Zinc protoporphyrin IX (10).** Red powder purchased from Aldrich (Milwaukee, WI, USA).

**Chlorin-*e*6 (11).** Brownish-green powder purchased from Porphyrin Products (Logan, UT, USA).

**Chlorin-*e*6 trimethyl ester (12). $C_{37}H_{42}N_4O_6$**. A solution of **11** (50 mg) was methylated using diazomethane at 0 °C for 15 min, providing 48 mg dark green powder (96% yield). Mp 205–207 °C (lit.[4,17] 207–208 °C); $^1$H NMR data were consistent with literature values;[4,7] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 403 (91,207); 501 (8942); 531 (4322); 609 (4089); 665 (28,071); FAB-MS: *m*/*z* 639 $(M+H)^+$.

**Zinc chlorin-*e*6 trimethyl ester (13). $C_{37}H_{40}N_4O_6Zn$.** Compound **12** (20 mg) was refluxed with zinc(II) acetate in toluene for 2 h, washed with water and dried over

sodium sulfate, providing 6 mg dark green powder (30% yield). Mp 130 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.53 (1H, s), 9.48 (1H, s), 8.54 (1H, s), 8.05 (1H, dd, $J$ = 18, 2 Hz), 6.18 (1H, dd, $J$ = 17, 2 Hz), 6.03 (1H, dd,$J$ = 17, 2 Hz), 5.20 (2H, q), 4.35 (1H, m), 4.29 (3H, s), 4.20 (1H, m), 3.80 (3H, s), 3.70 (2H, q), 3.58 (3H, s), 3.44 (3H, s), 3.33 (3H, s), 3.28 (3H, s), 2.55 (2H, m), 2.25 (2H, m), 1.80 (3H, m), 1.70 (3H, m); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (186,320); 512 (7842); 596 (12,635); 639 (73,915); FAB-MS: $m/z$ 702.4 $(M+H)^+$.

**13$^2$(*R*)-Hydroxy pheophorbide-*a* methyl ester (14). $C_{36}H_{38}N_4O_6$.** Compound **14** was prepared by a literature procedure,[7] providing 80 mg brownish-green powder (80% yield). Mp 233 °C (lit.[17] > 300 °C); $^1$H NMR data were consistent with literature values;[1] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 415 (48,498); 506 (5363); 536 (4220); 611 (3668); 669 (23,066); FAB-MS: $m/z$ 623 $(M+H)^+$.

**Pyropheophorbide-*a* methyl ester (15). $C_{34}H_{36}N_4O_3$.** Compound **15** was prepared by a literature procedure,[7] providing 27 mg brown powder (90% yield). Mp 233 °C (lit.[17] 217–219 °C); $^1$H NMR and UV–vis data were consistent with literature values;[17] FAB-MS: $m/z$ 549 $(M+H)^+$.

**13$^1$-Deoxypyropheophorbide-*a* methyl ester (16). $C_{34}H_{38}N_4O_2$.** Compound **16** was prepared by a literature procedure,[17] providing 12 mg brown powder (60% yield). Mp 170 °C [lit.[18] 180–182 °C]; $^1$H NMR data were consistent with literature values;[1,18] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 403 (81,423); 503 (8,821), 593 (3,158), 648 (18,184); FAB-MS: $m/z$ 536 $(M+H)^+$.

**Pheophorbide-*a* benzyl ester (17). $C_{42}H_{42}N_4O_5$.** Compound **2** (10 mg) was stirred with di-*tert*-butyl dicarbonate (1 equiv, 0.16 mmol) and 2 mg of dimethylaminopyridine for 10 min. Then, benzyl alcohol (1 equiv, 0.16 mmol) was added and stirring continued for 1 h. The product was purified using silica gel column chromatography, eluting with $CHCl_3$/hexane/EtOAc (1:3:2), providing 3 mg brown powder (30% yield). Mp 118 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.54 (1H, s), 9.40 (1H, s), 8.58 (1H, s), 8.0 (1H, dd, $J$ = 18, 2 Hz), 7.30 (5H, m), 6.30 (1H, dd, $J$ = 17, 1.3 Hz), 6.27 (1H, s), 6.19 (1H, dd, $J$ = 11.7, 1.3 Hz), 5.30 (2H, s), 4.48 (1H, dq, $J$ = 1.8, 7, 7, 1.8 Hz), 4.25 (1H, m), 3.90 (3H, s), 3.70 (3H, s), 3.67 (2H, q), 3.40 (3H, s), 3.22 (3H, s), 2.58 (2H, m), 2.29 (2H, m), 1.79 (3H, d), 1.71 (3H, t), 0.81 (1H, br s), 1.70 (1H, s); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (84,438); 507 (9787), 538 (8904), 610 (7695), 668 (38,210); FAB-MS: $m/z$ 684 $(M+H)^+$.

**Pheophorbide-*a* ethyl ester (18). $C_{37}H_{40}N_4O_5$.** Compound **18** was prepared as described above for **17**, but using ethyl alcohol (30% yield). Mp 155 °C; $^1$H NMR data were consistent with literature values;[19] UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (84,438); 507 (9787), 538 (8904), 610 (7695), 668 (38,210); FAB-MS: $m/z$ 622 $(M+H)^+$.

**Pheophorbide-*a* butyl ester (19). $C_{39}H_{44}N_4O_5$.** Compound **19** was prepared as described above for **17**, but using butyl alcohol (30% yield). Mp 110 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.47 (1H, s), 9.33 (1H, s), 8.49 (1H, s), 7.99 (1H, dd, $J$ = 18, 2 Hz), 6.30 (1H, dd, $J$ = 11.7, 2 Hz), 6.27 (1H, s), 6.19 (1H, dd, $J$ = 11.7, 2 Hz), 4.50 (1H, dq), 4.21 (1H, m), 3.90 (3H, s), 3.80 (3H, s), 3.62 (2H, q), 3.34 (3H, s), 3.18 (3H, s), 3.09 (2H, m), 2.58 (2H, m), 2.29 (2H, m), 1.79 (3H, t), 1.71 (3H, t), 1.50 (4H, m), 1.10 (3H, t), 0.81 (1H, s), 1.70 (1H, s); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (84,438); 507 (9787), 538 (8904), 610 (7695), 668 (38,210); FAB-MS: $m/z$ 649 $(M+H)^+$.

**Pheophorbide-*a* hexyl ester (20). $C_{41}H_{48}N_4O_5$.** Compound **20** was prepared as described above for **17**, but using hexyl alcohol (30% yield). Mp 175 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.53 (1H, s), 9.39 (1H, s), 8.56 (1H, s), 7.9 (1H, dd, $J$ = 18, 2 Hz), 6.30 (1H, dd, $J$ = 12, 1.3 Hz), 6.24 (1H, s), 6.19 (1H, dd, $J$ = 11.7, 1.3 Hz), 4.47 (1H, m), 4.21 (1H, m), 3.88 (3H, s), 3.82 (2H, q), 3.66 (3H, s), 3.40 (2H, m), 3.38 (3H, s), 3.24 (3H, s), 2.58 (2H, m), 2.40 (2H, m), 1.80 (3H, t), 1.71 (3H, d), 1.54 (4H, m), 1.34 (4H, m), 1.10 (3H, t), 0.81 (1H, s), 1.70 (1H, s); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (84,438); 507 (9787), 538 (8904), 610 (7695), 668 (38,210); FAB-MS: $m/z$ 677.6 $(M+H)^+$.

**Pheophorbide-*a* octyl ester (21). $C_{43}H_{52}N_4O_5$.** Compound **21** was prepared as described above for **17**, but using octyl alcohol (30% yield). $^1$H NMR (300 MHz, $CDCl_3$): δ 9.51 (1H, s), 9.38 (1H, s), 8.55 (1H, s), 7.90 (1H, dd, $J$ = 18, 2 Hz), 6.25 (1H, s), 6.20 (1H, dd, $J$ = 11.7, 1.3 Hz), 6.19 (1H, dd, $J$ = 11.7, 1.3 Hz), 4.50 (1H, m), 4.10 (1H, dq, $J$ = 7, 1.8 Hz), 3.90 (3H, s), 3.74 (3H, s), 3.62 (2H, q), 3.45 (2H, m), 3.34 (3H, s), 3.18 (3H, s), 2.58 (2H, m), 2.29 (2H, m), 1.79 (3H, d), 1.71 (3H, t), 1.70 (12H, m), 0.90 (3H, m), 0.81 (1H, s), 1.70 (1H, s); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 413 (84,438); 507 (9787), 538 (8904), 610 (7695), 668 (38,210); FAB-MS: $m/z$ 705 $(M+H)^+$.

**Zinc 13$^2$(*R*)-hydroxy pheophorbide-*a* methyl ester (22). $C_{36}H_{36}N_4O_6Zn$.** Compound **14** (20 mg) was refluxed with zinc(II) acetate (1 equiv) in toluene for 2 h, washed with water and the organic solvent was dried over anhydrous sodium sulfate. Silica gel column chromatography was used for purification, eluting with $CH_2Cl_2$/hexane/EtOAc (2:2:1) to obtain 6 mg product (30% yield). Mp 114 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.76 (1H, s), 9.55 (1H, s), 8.70 (1H, s), 8.01 (1H, dd, $J$ = 18, 2 Hz), 6.34 (1H, dd, $J$ = 12, 2 Hz), 6.18 (1H, dd, $J$ = 12, 2 Hz), 5.30 (1H, s), 4.45 (1H, m), 4.25 (1H, m), 3.89 (3H, s), 3.75 (2H, q), 3.53 (3H, s), 3.44 (3H, s), 3.27 (3H, s), 3.18 (3H, s), 2.30 (4H, m), 1.79 (3H, t), 1.71 (3H, t); UV–vis ($\lambda_{max}$, ε) ($CHCl_3$): 416 (141,592); 521 (7644); 568 (13,948); 609 (21,221); 657 (71,358); FAB-MS: $m/z$ 684 $(M+H)^+$.

**Nickel pyropheophorbide-*a* methyl ester (23). $C_{34}H_{34}N_4O_3Ni$.** Compound **15** (30 mg) was refluxed with nickel(II) acetate in toluene for 2 h. The product was washed with water and dried over anhydrous sodium sulfate. The desired product was purified using silica gel column chromatography, eluting with $CH_2Cl_2$/hexane/EtOAc (2:2:1), providing 9 mg (30% yield). Mp 171–172 °C; $^1$H NMR (300 MHz, $CDCl_3$): δ 9.39 (1H, s), 9.18 (1H, s), 8.29 (1H, s), 7.90 (1H, dd, $J$ = 18, 2 Hz),

6.30 (1H, dd, $J=12$, 2 Hz), 6.10 (1H, dd, $J=12$, 2 Hz), 4.94 (2H, m), 4.48 (1H, dq, $J=7$ Hz, 1.8 Hz), 4.25 (1H, m), 3.74 (2H, m), 3.70 (3H, s), 3.60 (3H, s), 3.29 (3H, s), 3.23 (3H, s), 2.58 (2H, m), 2.29 (2H, m), 1.79 (3H, d), 1.73 (3H, t); UV–vis ($\lambda_{max}$, $\varepsilon$) ($CHCl_3$): 420 (59,552); 651 (53,959); FAB-MS: $m/z$ 605 $(M+H)^+$.

**In vitro cytotoxicity assay.** The in vitro cytotoxicity assay followed the procedure described by Rubinstein et al.[20] Test compounds were prepared in DMSO and stored at −80 °C to prevent the degradation and the loss of biological activity that was found to occur upon storage at −20 °C. The final solvent concentration in cell culture was less than 2% v/v of DMSO, a concentration without effect on cell replication. The human tumor cell line panel included human oral epidermoid carcinoma (KB, ATCC # CCL17), multi-drug resistant (MDR) KB sub-line (KB-VCR), multi-drug resistance associated protein (MRP) KB sub-line (KB-7d), and malignant melanoma (SK-MEL-2, ATCC # HTB68). Human embryo lung fibroblast (HEL, ATCC # CCL137) was also included as an example of a non-tumorgenic human cell line. Drug-resistant cell lines were a generous gift of Dr. Y. C. Cheng (Yale University) and were described in detail.[21] Cells were incubated in RPMI-1640 with 100 μg/mL kanamycin and 10% v/v fetal bovine serum in a 5% $CO_2$ humidified incubator at 37 °C. Initial seeding densities varied between cell lines in order to ensure a final absorbance reading in the range 1–2.5 $A_{562}$ units. Cells were plated and treated with compounds for 24 h, photo-irradiated using a 25W incandescent lamp at a distance of 30 cm for 5 min and repeating exposure the next day. For treatment in dark, plates were covered by aluminum foil during incubation. After incubation for 3 days in total, the $ED_{50}$ value, the concentration that reduced the absorbance by 50%, was interpolated from dose–response graphs plotted using Prism™ (Graph Pad Inc., San Diego, CA, USA). Each test was performed in triplicate and experimental variation was assessed using at least three independent experiments.

### Gel lysis assay

The procedure used KB cells and was based on the method of Bastow et al.[22] Cells were plated at $2\times10^4$/cm$^2$ overnight. After discarding the medium, test compounds (concentration at 5-fold of $ED_{50}$ values) were added in fresh medium and incubation was continued overnight. Cultures were exposed to visible light using a 25 W incandescent lamp at a distance of 30 cm for 5 min. After 3 h of incubation, cells were scraped into ice-cold PBS and pelleted by centrifugation ($5\times100g$, 5 min). Washed cell pellets were resuspended in 80 μL of a 37 °C gel solution (1% w/v low-melting point agarose gel in PBS), cast in a mold and refrigerated for 5 min to form $6\times7\times2$ mm agarose plugs. Plugs of cells were incubated in lysis-digestion buffer (0.4 M EDTA, 0.01 M Tris–HCl, pH 8.0, 1% w/v *N*-lauryl-sarcosine and 100 mg/mL proteinase K) at 43 °C overnight. The treated plugs were equilibrated in TBE buffer (10 min, ambient temperature) and placed against a gel comb in a horizontal gel cast tray [BRL Inc. (Bethesda, MD, USA), H4 system format]. After pouring a 55 °C gel solution containing 0.8% w/v agarose in TBE and allowing it to gel, electrophoresis was carried out at 3.5 V/cm (measured between electrodes) at 4 °C for 16 h without buffer recirculation. The gel were stained with 1 μg/mL ethidium bromide in water, treated overnight at 25 °C with RNAase A (1 μg/mL), and then photographed under ultraviolet illumination. A representative gel is shown in Figure 1. Semi-quantitative analysis of double-stranded DNA breaks was done by densitometry using a STORM phosphorimager and ImageQuant 5.0 software (Molecular Dynamics Inc., Sunnyvale, CA, USA).

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