**Cytotoxic compounds from the leaves of *Combretum paniculatum* Vent**

Abimbola Sowemimo1*, Maryna van de Venter2, Lucinda Baatjies2, Trevor Koekemoer2, Saburi Adesanya3 and Wenhan Lin4

1Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, College of Medicine Campus, Idi-Araba, Lagos State, Nigeria.

2Department of Biochemistry and Microbiology, P.O Box 77000, Nelson Mandela Metropolitan University, Port Elizabeth 6031, South Africa.

3Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria.

4Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, China.

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*Corresponding author. E-mail: asowemimo@unilag.edu.ng. Tel: +234 803 936 3361.

**INTRODUCTION**

*Combretum paniculatum* Vent. (Combretaceae), a scandent shrub or robust liane with vivid scarlet flowers attaining 15 m length is widespread in tropical Africa. It is used locally in the treatment of carcinomous tumors. The cytotoxic activity of pheophorbide a and pheophorbide a-methyl ester isolated from the leaves of *C. paniculatum* were investigated. *In vitro* cytotoxicity of the compounds were evaluated against HT-29, MCF-7 and HeLa cancer cell lines using the methyl thiazolyl tetrazolium (MTT) assay and peripheral blood mononuclear cells. Cell cycle analysis was performed using propidium iodide staining and flow cytometry. Pheophorbide a was more toxic to MCF-7 and HeLa cells than its methyl ester, while the opposite result was seen in HT-29 cells. The methyl ester killed all the peripheral blood mononuclear cells from healthy donor blood while less inhibition was observed with pheophorbide a. Both compounds induced cell cycle arrest in HeLa cells in the G0/G1 phase, with pheophorbide a-methyl ester yielding a more enhanced G0/G1 arrest. After 24 h of exposure, the percentage of HeLa cells in the G0/G1 phase was 38.5% in vehicle control cells, 55.9% in pheophorbide a and 70.0% in pheophorbide a-methyl ester treated cells. The *in vitro* effect of *C. paniculatum* on cancer cell lines may be ascribed to the presence of pheophorbide a and its methyl ester. They exert this effect through the induction of cell cycle arrest in the G0/G1 phase of the cell cycle.

Key words: Cytotoxicity, cancer, *Combretum*, pheophorbide a, pheophorbide a-methyl ester.
2011). This present work reports the isolation and cytotoxic activity of two compounds (pheophorbide a and pheophorbide a-methyl ester) isolated from the leaves of *C. paniculatum* against colon (HT-29), breast (MCF-7) and cervical (HeLa) cancer cell lines in vitro, peripheral blood mononuclear cells (PBMCs) and their effect on the cell cycle.

**MATERIALS AND METHODS**

Leaves of *C. paniculatum* Vent. (Combretaceae) were collected from the Oko Okeki Forest Reserve, Nigeria in July 2008 and authenticated by comparison with corresponding herbarium specimens at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where a voucher specimen (FHI 107980) was also deposited. The plant was air dried for two days followed by drying in a hot air oven at 40°C and ground to powder.

**Extraction and isolation**

The powdered plant material (1 kg) was macerated with ethanol (3 L) at room temperature for 72 h. The ethanolic extract was concentrated *in vacuo* to get a dark green residue (24.8 g). This residue was suspended in water and partitioned successively with petroleum ether, EtOAc and n-BuOH. The yield was 9.5, 1.2 and 0.8 g, respectively. The EtOAc fraction was subjected to vacuum liquid chromatography (VLC) gradient elution with petroleum ether and ethyl acetate which gave 6 fractions. Fractions 3 (90 mg) and 4 (96 mg) were further subjected to column chromatography over silica gel eluting with petroleum ether : EtOAc (7:3) and (2:1), respectively, to yield C-1 (5 mg), a light green solid and C-2 (4 mg), a light green solid respectively.

**Cytotoxicity assay**

Cervical (HeLa), colorectal (HT-29) and breast (MCF-7) cancer cells were seeded at 6,000 cells/well in 96-well plates and left to attach overnight at 37°C in a humidified incubator and 5% CO₂. PBMCs (peripheral blood mononuclear cells) were isolated from venous blood of a healthy donor using heparinized Vacutainer preparation tubes (Beckton Dickinson, Plymouth, UK) within 30 min of collection. PBMCs were seeded at 100,000 cells/well in round bottomed 96-well plates. The isolated compounds were resuspended in DMSO (0.25%), sonicated for 15 min, and complete RPMI-1640 medium was added to reach concentrations of 62.5 to 250 μg/ml. Cisplatin (10 and 100 μM) was used as the reference drug. The cells were treated for 48 h after which the medium was replaced with 200 μl MTT (Sigma) (0.5 mg/ml in RPMI 1640:10% FBS). After a further 4 h incubation at 37°C, the MTT was removed and the purple formazan product was dissolved in DMSO and absorbance measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). CellTiter-Blue® assay (Promega) was performed according to manufacturer’s instructions for the PBMCs and fluorescence was read at 544 em/590 nm using a Fluoroskan Ascent FL fluorometer (ThermoLabsystems, Finland). All experiments were performed in quadruplicate.

**Cell cycle analysis**

HeLa cells were seeded into 10 cm cell culture dishes (Nunc) at 1.15 x 10⁵ cells per ml and incubated in a 37°C incubator supplemented with 5% CO₂ for 24 h. The isolated compounds (62.5 μg/ml) or cisplatin (50 μM) were added and the cells were incubated for a further 24 and 48 h. DNA cell cycle analysis was performed using the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter). The assay was carried out as per kit instructions and the results were analyzed on a Beckman Coulter FC500 flow cytometer. MultiCycle software for cell cycle analysis was used to calculate the percentage of cells in each of the cell cycle phases.

**RESULTS AND DISCUSSION**

The phytochemical investigation of the leaves of *C. paniculatum* gave two known compounds, pheophorbide a (C-1) and pheophorbide a-methyl ester (C-2) established with the aid of NMR spectroscopic techniques in addition to comparison with data found in literature (Nozawa et al., 1992; Ohsima et al., 1994; Rho et al., 2003). The comparative growth inhibition results (Figure 1) suggest that at 62.5, 125 and 250 μg/ml, pheophorbide a and pheophorbide a-methyl ester were most effective against MCF-7 breast and HeLa cervical cancer cell lines. In the HT-29 colon cancer cell line, pheophorbide a showed more than 50% inhibition only at 250 μg/ml, while pheophorbide a-methyl ester showed more than 50% inhibition at 125 and 250 μg/ml. Pheophorbide a was however more active than pheophorbide a-methyl ester in the other two cell lines tested. The results from the CellTiter Blue® assay on the PBMCs (Figure 2) suggested that pheophorbide a is less toxic than its methyl ester to normal cells. HeLa cells were used to establish whether the two purified compounds induced cell cycle arrest. Cisplatin at 50 μM typically caused an accumulation of the cells in the S phase after 48 h of treatment (Table 1). A large increase in the size of the sub-G₁ peak, corresponding to apoptotic cells, was seen after 24 h of cisplatin treatment and the effect was more pronounced after 48 h. The two isolated compounds both induced cell cycle arrest in the G₁/G₀ phase. Although, pheophorbide a was more toxic to HeLa cells, the methyl ester caused a more enhanced G₁/G₀ arrest on the cell cycle after 24 h. The percentage of HeLa cells in the G₁/G₀ phase was 38.5% for the vehicle control, 55.9% for the pheophorbide a and 70.0% for the pheophorbide a-methyl ester treated cells (Table 1). After 48 h of treatment, about 80% of the cells were arrested in G₁/G₀ with both compounds. Interestingly, the sub-G₁ peak did not increase with either of the two compounds, even after 48 h. Data demonstrating the anti-tumor activity of chlorophyll related compounds are not too common. However, pheophorbide a isolated from *Clerodendrum calamitosum* has been reported to display both strong and broad activity against epidermoid carcinoma of the nasopharynx (KB), ovarian carcinoma (1A9), kidney carcinoma (CA KI-1), malignant melanoma (SK-MEL-2), ileocecal carcinoma...
Inhibition (%)

Figure 1. Screening results of compounds in MCF-7, HT-29 and HeLa cancer cells. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control.

Inhibition (%)

Figure 2. Percentage inhibition of compounds on PBMCs. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control. Pheophorbide a, C-1; pheophorbide a-methyl ester, C-2.

(HCT-8), human lung cancer (A549) and breast adenocarcinoma (MCF-7) (Cheng et al., 2001), while pheophorbide a-methyl ester from Garuga pinnata has been reported to show photo-dependent cytotoxic activity (Kashiwada et al., 1997).

Most previous studies have reported on the photodynamic activities of pheophorbide a and its derivatives (Tang et al., 2006) and not on its cytotoxic activity and mechanism of action in the absence of photo-irradiation. In a recent study by Mohan et al. (2010), fractions of an extract from Typhonium flagelliforme were shown to induce G1/G0 arrest and apoptosis in CEMss human T4-lymphoblastoid cells. The most active fraction contained, amongst other compounds, four pheophorbide related
Table 1. Effect of pheophorbide a and its methyl ester on the cell cycle distribution of HeLa cells after 24 and 48 h of exposure.

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Treatment</th>
<th>G1/G0 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Vehicle control</td>
<td>38.5</td>
<td>48.6</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>34.4</td>
<td>41.8</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>C-1 (pheophorbide a)</td>
<td>55.9</td>
<td>38.0</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>C-2 (pheophorbide a-methyl ester)</td>
<td>70.0</td>
<td>6.2</td>
<td>23.9</td>
</tr>
<tr>
<td>48</td>
<td>Vehicle control</td>
<td>59.0</td>
<td>21.5</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>42.7</td>
<td>35.1</td>
<td>22.2</td>
</tr>
<tr>
<td></td>
<td>C-1 (pheophorbide a)</td>
<td>78.2</td>
<td>13.8</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>C-2 (pheophorbide a-methyl ester)</td>
<td>83.1</td>
<td>9.0</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Vehicle control, cells treated with 0.125% DMSO; 50 µM cisplatin is the positive control; G1/G0, cells in the G1 and G0 phase of the cell cycle; S, cells in the synthesis phase of the cell cycle; G2/M, cells in the G2 and M phase of the cell cycle.

The cell cycle analysis results revealed that the two compounds act in a very similar way through induction of cell cycle arrest in the G1/G0 phase. No increase was observed in the sub-G1 (apoptotic) peak and therefore, it appeared that the reduction in viable cell numbers as compared to vehicle control treated cells was due to inhibition of proliferation rather than cytotoxicity. This is the first report on the isolation of the two compounds, pheophorbide a and its methyl ester from *C. paniculatum* and the induction of cell cycle arrest in HeLa cells in the absence of photoactivation. Our results further suggested that pheophorbide a is a more potent inhibitor of proliferation than its methyl ester in at least two of the three cell lines investigated. Interestingly, the methyl ester derivative was more cytotoxic to PBMCs isolated from normal human blood.

The results obtained in this work further support the traditional use of the plant in the treatment of cancer.

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