Plaunotol from *Croton stellatopilosus* Ohba inhibited cell growth and induced apoptosis in human cancer cell lines

Charoenwong Premprasert, Supreeya Yoenyongsawad, Supinya Tewtrakul, and Juraithip Wungsintaweeekul*

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand

Received: 6 November 2017; Revised: 5 March 2018; Accepted: 10 April 2018

Abstract

Plaunotol is an acyclic diterpene alcohol that was evaluated for anti-proliferative activity in four human cancer cell lines: HeLa; HT-29; MCF-7; and KB. After the cells were treated with plaunotol, cell viability was determined using the MTT assay. The results showed that plaunotol inhibited the growth of human cancer cell lines with an IC₅₀ of 65.47±6.39 μM (HeLa), 72.92±5.73 μM (HT-29), 80.90±3.48 μM (KB), and 62.25±9.15 μM (MCF-7). For apoptotic detection, the cells treated with plaunotol were stained with Annexin-V/7-AAD reagent. The results indicated that plaunotol induced cell death or apoptosis. The transcription profile of apoptotic-associated genes, including *TNF*-α, *BCL*-2, *BAX*, and *BAK* genes, was determined using the qRT-PCR technique and the expression level was calculated as the relative quantitation (RQ). The ratio between RQ of *BCL*-2 and *BAX* suggested that plaunotol significantly induced apoptosis in HeLa, MCF-7, and HT-29 cell lines, in particular.

Keywords: plaunotol, anti-proliferative activity, MTT assay, apoptosis, human cancer cell lines

1. Introduction

Plaunotol is an acyclic diterpene alcohol derived from geranylgeranyl diphosphate (Figure 1). Plaunotol was first isolated in 1978 and was found only in *Croton stellatopilosus* Ohba or plaunoi (Thai name) (Ogiso *et al.*, 1978). Plaunotol was claimed to have anti-peptic ulcer ability and it was successfully registered with the World Health Organization (WHO) (Ogiso *et al.*, 1985). It was further processed into a soft gelatin capsule under the trade name Kelna® by Sankyo Daiichi, Japan. Recently, the partially purified plaunotol extract was reported to be potentially safe after acute and chronic oral toxicity tests in an animal model (Chattham, Chivapat, Chaikitwattana, & De-Eknamkul, 2013).

Plaunotol has a gastro-protective effect that mediates several mechanisms such as releasing prostaglandins (PGE₂ and PGI₂) and secretin as well as inhibiting neutrophil activation (Murakami *et al.*, 1999; Shiratori, Watanabe, & Takeuchi, 1993; Ushiyama *et al.*, 1987). Considering *Croton* species, some diterpenoids were reported to have anticancer and antitumor activities such as trans-dehydrocrotonin and *trans*-crotonin from *C. cajucara* (Grynberg *et al.*, 1999), neocrotocembranal from the stem bark of *C. oblongifolius* (Roengsumran *et al.*, 1999), and ent-15-oxo-kaur-16-ene-18-oic acid from the bark of *C. argyrophylloides* (Santos *et al.*, 2009). Nowadays, several secondary metabolites with interesting biological activities serve as leading compounds in the development of drugs such as paclitaxel and docetaxel. Both drugs are strong anticancer agents and are already being used in the treatment of cancer. Interestingly, other isoprenyl molecules such as farnesol (Joo, Liao, Collins, Grissom, & Jett, 2007; Park *et al.*, 2014), geranylgeraniol (GGOH) (Ohizumi *et al.*, 1995; Yoshikawa *et al.*, 2009), geranylgeranoic (Nakamura *et al.*, 1996; Shidoji *et al.*, 2006; Shidoji, etc.)

*Corresponding author
Email address: juraithip.w@psu.ac.th

**Figure 1.** Chemical structure of plaunotol.
2. Materials and Methods

2.1 Isolation of plaunotol

Plaunotol was extracted from *C. stellatopilosus* leaves using silica gel column chromatography described by Premprasert et al. (2013). The structure was confirmed by spectroscopic methods [1H-, 13C-NMR, and MS].

Plaunotol: a pale yellow oil; IR νmax cm⁻¹ 3300 (O-H), 1665 (C=O), 1440 (C-H), 1380, 1000; 1H NMR (CDCl₃, 500 MHz) δ: 1.60 (3H, s, H-19), 1.60 (3H, s, H-20), 1.64 (3H, s, H-16), 1.64 (3H, s, H-17), 1.95 (2H, m, H-13), 2.02 (2H, m, H-12), 2.02 (2H, m, H-4), 2.10 (2H, m, H-8), 2.16 (2H, m, H-5), 4.05 (2H, s, DNA), 4.07 (2H, d, J = 7.1 Hz, H-1), 5.06 (1H, m, H-10), 5.09 (1H, m, H-14), 5.22 (1H, m, H-6); 13C NMR (CDCl₃, 125 MHz) δ: 123.9 (C-10), 124.0 (C-14), 124.2 (C-2), 127.4 (C-6), 131.3 (C-11), 135.3 (C-15), 138.8 (C-7), 138.9 (C-3), 15.9 (C-20), 16.4 (C-17), 17.6 (C-19), 25.6 (C-16), 25.8 (C-5), 26.6 (C-13), 26.7 (C-9), 34.8 (C-8), 39.2 (C-4), 39.6 (C-12), 58.9 (C-18), 59.8 (C-1); EI-MS at m/z 306.255 (M⁺).

2.2 Chemicals

All chemicals used in this study were of analytical grade. Dulbecco’s Modified Eagles Medium (DMEM), trypan blue, trypsin-EDTA, fetal bovine serum (FBS), and MTT; 3-(4, 5-dimethyl-2-thiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide were from Gibco® BRL, California, USA. The antibiotics (penicillin G [100 U/mL] plus streptomycin [100 μg/mL]) were supplied by Invitrogen®, California, USA. The Total RNA mini kit used for total RNA isolation was from Geneaid®, New Taipei City, Taiwan. TaKaRa One Step SYBR® PrimerScript™ RT-PCR Kit II was purchased from Takara Bio Inc., Japan. Primers used for qRT-PCR were designed from the Gene Bank information of *Homo sapiens* (http://ncbi.nlm.nih.gov). Assay kits; Muse™ Annexin-V et Dead cell reagent and Muse™ cell cycle reagents were purchased from Merck, Darmstadt, Germany.

2.3 Cell lines

Cells, including human breast carcinoma cell line (MCF-7; CLS No. 300273), human cervical carcinoma cell line (KB, CLS No. 300446), human cervix adenocarcinoma (HeLa, CLS No. 300194), and human colon adenocarcinoma (HT-29, CLS No. 300215) were obtained from the Cell Line Service, Heidelberg, Germany. The human gingival fibroblast (HGF) cell line was kindly provided by the Faculty of Dentistry, Prince of Songkla University. Cells were maintained in DMEM supplemented with 10% FBS and 2% antibiotics (penicillin and streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. The monolayered cells were sub-cultured weekly with 0.25% trypsin-EDTA until they reached 80% confluence.

2.4 Cell viability assay

Cell viability was evaluated by the MTT assay with some modifications (Mosmann, 1983). The cells were washed twice with PBS briefly then harvested using trypsin-EDTA. The harvested cells (1×10⁵ cells per well) were each placed in a well of a 96-well plate. These cells were attached to the plate after 24-h incubation at 37 °C in an atmosphere of 5% CO₂. Aliquots of medium containing different concentrations of plaunotol (3, 10, 30, and 100 μM) were added and the cell cultures were incubated for 48 h. This experiment established that the use of DMSO concentrations (0.2 % v/v) in the cell cultures caused no cell damage. After the incubation period, the culture medium was removed and washed twice with PBS. Then 100 μL of MTT reagent (5 mg/mL in PBS) was added to each well and the cells were incubated for 3 h. Cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt to formazan crystal. The obtained formazan crystals were dissolved in acidic condition (100 μL of 0.1 N HCl in isopropyl alcohol). The absorbance of the samples was analyzed with a microplate reader (DTX 880 multimode detector, Beckman Coulter Inc, Austria) using a
test wavelength of 570 nm. The correlation between concentration and % inhibition was interpreted at 50% inhibitory concentration (IC50) values in micromolar units.

2.5 Apoptosis detection

To evaluate the apoptotic activity of plaunotol, Annexin-V and 7-AAD double staining was carried out as described by Vermes, Haanen, Steffens-Nakken, H. & Reutelingsperger (1995). The human cancer cell lines, i.e. HeLa, HT-29, KB, and MCF-7, were separately seeded briefly at 5×102 cells per well and treated with 75 µM and 150 µM of plaunotol for 48 h. Paclitaxel at a concentration of 1 µM was used as the positive control. After treatment, the cells were washed twice with PBS and harvested with 0.25% trypsin-EDTA, followed by centrifugation at 500g for 5 min. Then, the cell pellet was re-suspended in 100 µL of DMEM media containing 1% FBS followed by the addition of 100 µL of Muse™ Annexin V and 7-AAD. The solution was incubated for 20 min at room temperature under dark conditions. Finally, the stained cells were analyzed by flow cytometer using a Muse™ cell analyzer. The data was analyzed using Muse™ 1.4 software and the data were shown as a four-quadrant dot plot. The statistics on four cell populations were obtained and the populations in each quadrant predicted the apoptosis in live, early apoptosis, late apoptosis, and death, respectively.

2.6 Cell cycle analysis

HeLa, HT-29, KB, and MCF-7 cancer cells were seeded into a 6-well plate at 5×103 cells/well. The cells were then treated with various doses of plaunotol (25 µM to 100 µM) and with DMSO (0.2%) as a control group. The treated cells were incubated for 48 h. After incubation, the cells were harvested using trypsin-EDTA and centrifuged at 500g for 5 min. The cells were then washed with PBS and fixed with ice cold ethanol (70% in water). After ethanol fixation for 3 h at –20 °C, the cells were then centrifuged at 500g for 5 min. The ethanol was removed and the fixed cells were rinsed with PBS. The cell pellet was re-suspended in PBS (0.25 mL per 5×103 cells/well) and centrifuged after which the supernatant was discarded. The Muse™ cell cycle kit reagent (200 µL) was added and the cells were re-suspended and incubated for 30 min in the dark at room temperature. The stained cells were analyzed by flow cytometer (Muse™ cell analyzer). The DNA content was analyzed and the dot plot was recorded. A histogram of the DNA content index was generated and the percentage of cells in each cell cycle phase (G0/G1, S and G2/M).

2.7 Primers design

The sequences of genes including TNF-α, BCL-2, BAX, BAK, and GAPDH were retrieved from GenBank Database (https://www.ncbi.nlm.nih.gov/). Oligonucleotides with 20-24 bp and theoretical melting temperatures ranging from 60 to 64 °C were designed accordingly, using Primer3 Software (http://simgene.com/Primer3). The list of primers is shown in Table 1.

2.8 Quantitative real-time PCR (qRT-PCR)

The human cancer cell lines (5×105 cells per well) in the 6-well plate were treated with plaunotol at concentrations of 50 µM and 75 µM and incubated further for 48 h at 37 °C in a 5% CO2 humidified atmosphere. The cells were treated with 0.2% (v/v) DMSO and paclitaxel (1 µM) as the negative and positive controls, respectively. After incubation, the cells were washed with PBS and treated with trypsin-EDTA. The suspension of cells was harvested by centrifugation at 500g for 5 min. The supernatant was removed and the cell pellet was rinsed with PBS and stored at –80°C until use.

The total RNA was isolated from the cells using Total RNA Mini Kit (Geneaid, Taiwan) according to the manufacturer’s protocol. The quality and quantity of total RNA of the treated cells were evaluated by UV spectrophotometer (Thermo Scientific, USA). The mRNA levels of genes were determined using qRT-PCR (ABI Prism® 7300) in the presence of RNA template and One Step SYBR® PrimeScript™ RT-PCR kit II (Perfect Real Time, Takara, Japan). The qRT-PCR was carried out in a final volume of 20 µL reaction; with mixture containing 0.8 µL of each primer (forward & reward) (Table 1), 10 µL of 2x one step RT-PCR buffer IV, 0.4 µL of ROX dye (50x), 2 µL of RNA (20 ng) as a template and 0.8 µL of PrimeScript one step enzyme mix II, and analyzed by the Step One Plus™ Real-Time PCR System (Applied Biosystems, USA).

Table 1. Primers used in qRT-PCR experiment.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number* (location)</th>
<th>Sequence (5’→3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-TNFα</td>
<td>NM_000594.3</td>
<td>TGG TTG TTC CTC AGC TTC TTC TC</td>
<td>200</td>
</tr>
<tr>
<td>R-TNFα</td>
<td>(263-463)</td>
<td>AGG GTT TGC TAC AAC ATG GGC T</td>
<td></td>
</tr>
<tr>
<td>F-BCL2</td>
<td>NM_000633.2</td>
<td>CCT GTG GAT GAC TGA GTA CCT GTG</td>
<td>129</td>
</tr>
<tr>
<td>R-BCL2</td>
<td>(1015-1144)</td>
<td>CAC AGA AGA CCA GGA GAA ATC A</td>
<td></td>
</tr>
<tr>
<td>F-BAX</td>
<td>NM_001291428.1</td>
<td>GAG AGG TCT TTT TCC GAG TCG C</td>
<td>105</td>
</tr>
<tr>
<td>R-BAX</td>
<td>(335-440)</td>
<td>GCC TGG AGC ACC AGT TGG CTG</td>
<td></td>
</tr>
<tr>
<td>F-BAK</td>
<td>NM_001188.3</td>
<td>GAG AGG TCT TTT TCC GAG TGC C</td>
<td>159</td>
</tr>
<tr>
<td>R-BAK</td>
<td>(1366-1525)</td>
<td>GCC TGG AGC ACC AGT TGG CTG</td>
<td></td>
</tr>
<tr>
<td>F-GAPDH</td>
<td>NM_001256799.2</td>
<td>ACC CAC TCC TCC ACC TTT GAC</td>
<td>179</td>
</tr>
<tr>
<td>R-GAPDH</td>
<td>(1066-1245)</td>
<td>TCC TCT TGT GCT CTT GCT GG</td>
<td></td>
</tr>
</tbody>
</table>

and adjusted to the volume with RNase free water. At the optimal PCR condition, the amplification plot and melting curve were generated. The cycle number at threshold (set at 0.2) was recorded. The GAPDH gene was used as the endogenous gene. The expression level of the control treatment was used as a calibrator of each gene. The transcription profiles of the genes were expressed as relative quantitative (RQ), which was calculated using the comparative Ct method when \( RQ = 2^{\Delta \Delta Ct} \).

2.9 Statistical analysis

All data represented in this study are expressed as mean±SD. The experiments were performed in triplicate (n=3). Analysis of variance (ANOVA) followed by Dunnett’s post-test was used to determine the significant differences between the groups, and P values ≤0.05 and >0.01 were considered significant at 95% and 99% confidence. All statistical analyses were conducted using IBM SPSS version 22 for Windows software.

3. Results

3.1 Effect of plaunotol on cell viability

The MTT assay was performed to determine the cell viability of plaunotol in human cancer cell lines. The growth of human cancer cell lines was inhibited when cells were treated with plaunotol in a concentration-dependent manner. The relationship between cell viability and concentration yielded the IC\(_{50}\) value. Table 2 summarizes the IC\(_{50}\) of plaunotol and paclitaxel against the cell lines. It can be concluded that plaunotol exhibits a moderate anti-proliferative activity.

3.2 Effect of plaunotol on cell division

To understand the action of plaunotol on anti-proliferative activity, the effect of plaunotol on cell division was performed by staining the DNA with propidium iodide. The cell cycle assay, using flow cytometer, indicated that the proliferative activity, the effect of plaunotol on cell division was inhibited when cells were treated with plaunotol in a concentration.

3.3 Effect of plaunotol on apoptosis

In the treatment of the human cancer cell lines with plaunotol at 75 \( \mu \)M and 150 \( \mu \)M (equivalent to the IC\(_{50}\) and IC\(_{90}\), respectively) for 48 h, the cells were prepared for double staining according to the manufacturer’s protocol. After subjecting the mixture to the Muse™ analyzer, the population was gated. The results clearly showed that plaunotol altered the membrane from apoptosis (Figure 3A). This evidence appeared in each of the cancer cell types, although with different sensitivity. As shown in Figure 3B, plaunotol triggered apoptosis in the early phase. It can be noted that a high concentration of plaunotol had a cytotoxic effect on the HT-29.

3.4 Plaunotol caused apoptosis in both extrinsic and intrinsic pathways

After treating the human cancer cell lines with plaunotol (50 \( \mu \)M and 75 \( \mu \)M), the cells were harvested and the RNA extracted. The transcription profiles of apoptotic-associated genes, such as pro-apoptotic genes (TNF-α, BAX, and BAK) and anti-apoptotic genes (BCL2), were determined when GAPDH was an endogenous gene. The relative expression was calculated as the RQ value according to the equation in the experiment.

In consideration of the expression profile of TNF-α, treatment of the cells with plaunotol increased the expression of TNF-α in HeLa and HT-29 (Figure 4A). In contrast, the TNF-α mRNA levels in MCF7 and KB cells were suppressed after treating the cells with plaunotol. The expressions of the anti-apoptotic gene Bcl2 were decreased in each of the cell types. On the other hand, plaunotol did not affect BAX and BAK mRNAs in any of the cancer cell types. The ratio of expression level of BCL2 and BAX was estimated from the apoptotic-associate genes related to the apoptotic agent. The results in Figure 4B revealed that plaunotol caused apoptosis in HeLa, HT-29, and MCF7. In conclusion, plaunotol played an important role in altering cell division causing apoptosis by the suppression of apoptotic-associate genes via extrinsic and intrinsic pathways in HeLa, HT-29, and MCF7, but not in KB cells.

4. Discussion

Plaunotol or (E,Z,E)-7-hydroxymethyl-3,11,15-tri-methyl-2,6,10,14-hexadecatetraen-1-ol was registered with the World Health Organization (WHO) under the name of CS-684 in 1983. It was manufactured in the form of a soft-gelatin capsule (combined with corn oil) under the tradename of

<table>
<thead>
<tr>
<th>Compound (conc.)</th>
<th>IC(_{50}) in different cell line*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa HT-29 MCF7 KB</td>
<td></td>
</tr>
<tr>
<td>Plaunotol (( \mu )M)</td>
<td>65.5±6.4 72.9±5.7 62.3±9.1 80.9±3.5</td>
</tr>
<tr>
<td>Paclitaxel (nM)</td>
<td>12.3±2.9 7.6±4.0 4.5±2.5 11.4±0.5</td>
</tr>
</tbody>
</table>

*The IC\(_{50}\) on the human gingival fibroblast was > 100 \( \mu \)M.
Figure 2. Histograms of DNA content profiles in the HeLa, HT-29, MCF7, and KB cells after treatment at 75 µM and 100 µM of plaunotol in comparison with control and paclitaxel treatments for 48 h. Cells were stained with propidium iodide and analyzed by flow cytometry.

Figure 3. Percent of cell population of human cancer cell lines after treatment with different concentrations of plaunotol. Data are expressed as mean±SD of triplicate experiments and was analyzed by ANOVA followed by Dunnett’s post-test where ** was at P<0.001 and * was at P<0.05 when compared to control.
Figure 4A. Dot plots indicate amount of stained cells in each quadrant: live, early apoptosis, live cells, late apoptosis and dead cells, respectively in HeLa, HT-29, MCF7, and KB cells. Percentage of each quadrant indicates the population of cells obtained from gating.

Kelnac™ (Daiichi Sankyo Ltd., Tokyo, Japan). Since then, plaunotol has become an anti-peptic ulcer drug and is recommended by physicians to be combined with antibiotics and proton pump inhibitors for treatment of Helicobacter pylori-induced peptic ulcer (Takagi et al., 2000).

In the last three decades, pharmacological activities of plaunotol have been investigated extensively with various publications. It possesses several pharmacological activities, such as anti-inflammation, gastro-protection, antibacterial activity, and anticaner properties. Plaunotol induces the production of prostaglandins in gastric mucosa that results in the release of endogenous secretin (Shiratori et al., 1993) and suppresses the production of inflammatory mediators which are generated by leucocytes such as TNF-α and IL-8 (Murakami et al., 1999; Takagi et al., 2000). For anticaner activity, in particular, only a few reports on the effect of plaunotol in cancer cells were found. Plaunotol was reported to exert anti-cancer effects through its anti-angiogenic activity (Kawai et al., 2005) and it has direct effects on gastric and colon cancer cells (Yamada et al., 2007; Yoshikawa et al., 2009). Therefore, in the interest of increasing the value of C. stellatopilosus, we proceeded to demonstrate the efficiency of plaunotol for anti-proliferative and apoptosis activities.

The anti-proliferative activity of plaunotol in the present study was reported against four types of human cancers: MCF-7; KB; HeLa; and HT-29. In parallel, human gingival fibroblast cells were used to represent normal cells. Evaluation of the cytotoxic activity using the MTT assay revealed that plaunotol exhibited dose-dependent anti-proliferative activity against all human cancer cell lines and expressed an IC₅₀ ranging from 60 μM to 80 μM. In addition, at tested concentrations (<100 μM), plaunotol was not toxic to the HGF normal cell line. In DLD1-human colon adenocarcinoma, plaunotol and GGOH were shown to inhibit...
growth of the cancer cell by inducing caspase-mediated apoptosis. These results showed that plaunotol not only inhibited growth of HT-29, a human colon cancer cell, but also MCF-7, KB, and HeLa cells.

Several anti-cancer drugs, such as paclitaxel, are known to inhibit cancer growth and block the cell cycle (Payne & Miles, 2008). The cell cycle is the control process in eukaryotic cells which evaluates the condition of the genetic formation during cell division. Its mechanism is regulated by three internal checkpoints including the G0/G1 phase, S-phase, and G2/M phase. The G1 checkpoint is a major checkpoint caused by damage of DNA and the cell cannot pass to the next stage (S-phase). The S checkpoint checks the replication of DNA prior to undergoing the mitosis stage. The last stage, M checkpoint, evaluates whether all sister chromatids are correctly attached to spindle microtubules before the cell enters the irreversible anaphase. From the results of the study, the human cancer cell lines had different responses to plaunotol at 75 and 100 μM. Plaunotol exhibited anti-proliferative activity at the resting stage (G0/G1) of HeLa
Figure 5. The relative expression levels of apoptotic-associate genes in the human cancer cell lines (HeLa, HT-29, MCF-7, and KB cells) after treatment with plaunotol at 50 µM and 75 µM for 48 h. (A) the RQ values of TNF-α, BCL-2, BAX and BAK. (B) the RQ ratios of $RQ_{BCL-2}$ and $RQ_{BAX}$. 
and KB. On the other hand, it caused an inhibition during DNA synthesis (S) of MCF-7 and during cell division (G2/M) of HT-29. It can be concluded that plaunotol has anti-proliferative activity that affects cell division at different stages depending upon the type of cancer cell. The inhibitory effect of plaunotol against HeLa, HT-29, MCF-7, and KB-cells was confirmed by staining the cells with Annexin V. The results indicated that HeLa, HT-29, and MCF-7 were sensitive to plaunotol at 75 and 150 μM and induced apoptosis at the early stage of apoptosis. In contrast, plaunotol had less effect on KB-cells, which is a derivative of HeLa cells. A different mechanism of plaunotol on anti-proliferative activity in the KB-cells can be postulated.

Plaunotol has been reported to induce apoptosis of gastric cancer cells (Yamada et al., 2007). Moreover, it activated caspases and induced apoptosis in colon cancer (Yoshikawa et al., 2009). The present study clearly showed that plaunotol acted as an apoptotic agent in breast cancer, as well as cervix and colon cancers. It blocked the cell cycle during cell division, altered the cell membrane leading to cell death and inhibited BCL-2 expression which resulted in apoptosis of the cancer cells. In summary, plaunotol induces apoptosis through the death receptor and mitochondrial dependent pathway. In summary, the present study provided supportive data on the anti-proliferative activity and apoptotic mechanism of plaunotol in human cancer cells. Thus, plaunotol may have therapeutic potential in chemotherapy for the treatment of human cancer.

References


