Short communication

1’S-1’-Acetoxyeugenol acetate: A new chemotherapeutic natural compound against MCF-7 human breast cancer cells

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A R T I C L E I N F O

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A B S T R A C T

Medicinal plants containing active natural compounds have been used as an alternative treatment for cancer patients in many parts of the world especially in Asia (Itharat et al. 2004). In this report, we describe the cytotoxic and apoptotic properties of 1’S-1’-acetoxyeugenol acetate (AEA), an analogue of 1’S-1’-acetoxychavicol acetate (ACA), isolated from the Malaysian ethno-medicinal plant Alpinia conchigera Griff (Zingiberaeae) on human breast cancer cells. Data from MTT cell viability assays indicated that AEA induced both time- and dose-dependent cytotoxicity with an IC50 value of 14.0 μM within 36 h of treatment on MCF-7 cells, but not in HMEC normal control cells. Both annexin V-FITC/PI flow cytometric analysis and DNA fragmentation assays confirmed that AEA induced cell death via apoptosis. AEA was also found to induce cell cycle arrest in MCF-7 cells at the G0/G1 phase with no adverse cell cycle arrest effects on HMEC normal control cells. It was concluded that AEA isolated from the Malaysian tropical ginger represents a potential chemotherapeutic agent against human breast cancer cells with higher cytotoxicity potency than its analogue, ACA.

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Introduction

Alpinia conchigera, also known locally as ‘lengkuas ranting’, ‘lengkuas kecil’, ‘lengkuas padang’, ‘lengkuas getting’ or ‘chengke-nam’ (Janssen and Scheffer 1985) is a herbaceous perennial, 2–5 feet tall, found in eastern Bengal and southwards to Peninsular Malaysia and Sumatera (Burkill 1966). It is used as a condiment in the northern states of Peninsular Malaysia and occasionally in traditional medicine in the east coast to treat fungal infections. In Thailand, the rhizomes are used in traditional Thai medicine to relieve the gastro-intestinal disorders and in the preparation of Thai food dishes (Matsuda et al. 2005).

Traditional medicine from various plant types containing active natural compounds such as chalcones (Hsu et al. 2006), xanthoangelol (Tabata et al. 2005) and licochalcone-A (Fu et al. 2004) have all been reported as potential drugs for the treatment of cancer. In recent years, the pro-apoptotic effects of 1’S-1’-acetoxychavicol acetate (ACA) from the Thai ginger isolate, Languas galanga and Alpinia galanga, have been documented in human breast carcinoma cells (Campbell et al. 2007), human T cell lymphoma (Ichikawa et al. 2005) and in the inhibition of tumor-promoter-induced Epstein–Barr virus (Kondo et al. 1993). Even though previous studies have shown that ACA isolates from ginger exhibited anti-tumor properties against a wide variety of cancers, there have been no reports thus far on the cytotoxic and apoptotic effects of the closely related 1’S-1’-acetoxyeugenol acetate (AEA) (Fig. 1) on human breast cancer cells.

This study describes the purification, characterization and biological activity of six natural ACA analogs isolated from the rhizomes of the Malaysian wild-ginger, Alpinia conchigera (Zingiberaeae). We report herein the phytochemical data as well as our preliminary cytotoxic and apoptotic effects of AEA (Fig. 2) on MCF-7 human breast cancer cells.

Materials and methods

Plant material

Rhizomes of Alpinia conchigera Griff. were collected from Jeli province of Kelantan, East-coast of Peninsular Malaysia. The sample was identified by Prof. Dr. Halijah Ibrahim from the Institute of Biological Science, Faculty of Science, University Malaya. A voucher
specimen (KL5049) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University Malaya.

Reagents

RPMI-1640, MEGM, fetal bovine serum (FBS) and antibiotics were purchased from Lonza Inc. (USA). MTT reagent, Annexin V-FITC/PI apoptosis detection kit, propidium iodide (PI), paclitaxel, RNase A and Suicide Track™ DNA Ladder Isolation Kit were purchased from EMD Chemicals Inc. (Calbiochem, San Diego, CA, USA).

Extraction and isolation natural compounds

Air-dried and powdered rhizomes of *Alpinia conchigera* (2.1 kg) were extracted with dichloromethane at room temperature (72 h). The solvent was evaporated in vacuo to give dichloromethane extract. The extract was subjected to column chromatography (CC) on silica gel (Merck Kiesegel 60) with stepwise gradient of hexane-ethyl acetate. Fractions were collected separately and concentrated in vacuo at 40 °C. Fractions with similar profiles in TLC were pooled together to obtain six subfractions which were then subjected to further chromatographic analysis which yielded six compounds (1–6). The structures of compounds 1–6 were determined based on comparison of the $^1$H and $^{13}$C NMR data with those reported in the literatures (Lee and Ando 2001; Ando et al. 2005; Janssen and Scheffer 1985; Yang and Eilerman 1999; Barik et al. 1987; Mitsui et al. 1976). All spectral data were obtained on the following instruments; IR on the PerkinElmer FT-IR spectrometer RX1, UV on a Shimadzu UV-160A UV-Visible Recording Spectrophotometer, NMR on a JEOL (Japan Electronic Optics Laboratory Co. Ltd., Tokyo, Japan) JNM-LA400 FT-NMR spectrometer system (400 MHz) and MS on a Shimadzu GC-MS spectrometer (HP 6890 Series Mass Selective Detector and HP 6890 Series GC System).

LCMS analysis

LCMS analysis was done using Shimadzu LCMS-IT-TOF (Columbia, MD, USA) with a binary pump, an automatic injector and
MTT cytotoxic effects of ACA analogs from Alpinia conchigera on human breast cancer cells (MCF-7) and human normal breast cells (HMEC).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Compound</th>
<th>n</th>
<th>Treatment (h)</th>
<th>IC₅₀ value (µM)</th>
<th>Viability (% cells)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMEC</td>
<td>Paclitaxel (positive control)</td>
<td>3</td>
<td>36</td>
<td>n/a</td>
<td>95.2</td>
</tr>
<tr>
<td></td>
<td>1′S-1′-Acetoxychavicol acetate (ACA), 1</td>
<td>3</td>
<td>36</td>
<td>n/a</td>
<td>79.4</td>
</tr>
<tr>
<td></td>
<td>1′S-1′-Acetoxyeugenol acetate (AEA), 2</td>
<td>3</td>
<td>36</td>
<td>n/a</td>
<td>86.8</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Paclitaxel (positive control)</td>
<td>3</td>
<td>36</td>
<td>15.0</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>1′S-1′-Acetoxychavicol acetate (ACA), 1</td>
<td>3</td>
<td>36</td>
<td>20.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1′S-1′-Acetoxyeugenol acetate (AEA), 2</td>
<td>3</td>
<td>36</td>
<td>14.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1′-Hydroxychavicol acetate, 3</td>
<td>3</td>
<td>36</td>
<td>n/a</td>
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<tr>
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<td>trans-p-Coumaryl diacetate, 4</td>
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<td>36</td>
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<tr>
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<td>73.9</td>
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<tr>
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<td>p-Hydroxycinnamaldehyde, 6</td>
<td>3</td>
<td>36</td>
<td>n/a</td>
<td>70.6</td>
</tr>
</tbody>
</table>

³ Percentage of cell viability after 36 h incubation upon maximum treatment with 80.0 µM of each compound.

a photodiode array detector (SPD-M20A). Separations were carried out on a Waters XBridge C18 column (50 × 2.1 mm, 2.5 µm). A binary gradient solvent system of double-distilled water (eluent A)–acetonitrile (eluent B) was used as follows: 100% A and 0% B (0.0 min), 0% A and 100% B (6.0 min), 100% A and 0% B (8.5–10.0 min). Flow-rate at 0.5 ml/min was used and absorbance was detected at 254 nm. All tested solutions were filtered through Whatman 13 mm, 0.2-µm nylon membrane syringe filters before use.

Cell lines and cultivation conditions

Two human cell lines were used in this study, comprising of human breast adenocarcinoma (MCF-7) (CARIF, Malaysia) and human mammary epithelial cells (HMEC) (Lonza, USA), which was used as a normal cell control. MCF-7 cells were cultured as monolayers in RPMI-1640, supplemented with 10.0% (v/v) FBS, 100 U/ml penicillin and 100.0 µg/ml streptomycin, while HMEC cells were cultured in MEGM. All cultures were maintained at 37 °C in 5.0% CO₂ and 95.0% air.

MTT cell viability assay

Cytotoxic effects of all ACA analogues on MCF-7 and HMEC cells were determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye metabolism. All ACA analogues were dissolved in DMSO to a final concentration of 10.0 mM. Briefly, 2.0 × 10⁴ cells were treated in triplicates with each ACA analogue and paclitaxel. MTT reagent (5.0 mg/ml) was added and cells were incubated in the dark at 37 °C for 2-4 h. Formazan formation was detected at 254 nm. All tested solutions were filtered through Whatman 13 mm, 0.2-µm nylon membrane syringe filters before use.

Cell cycle analysis

Cells were seeded treated with AEA for 12 and 24 h before harvesting. Fixation was done with 70% (v/v) ethanol and incubated at −20 °C overnight. Fixed cells were washed with PBS and stained in 1.0 ml of PBS containing 50.0 µg/ml PI and 0.1 mg/ml RNase A. Fluorescence from a population of 2.0 × 10⁶ cells was detected using the BD FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA) and CellQuest Pro (IVD) software (Becton Dickinson, Mountain View, CA, USA).

Annexin V-FITC/PI analysis

Detection of apoptosis was conducted using the Annexin V-FITC/PI apoptosis detection kit according to manufacturer’s protocol. Briefly, media binding reagent containing FITC-conjugated annexin V anti-coagulant (200.0 µg/mg) was added to AEA treated MCF-7 and HMEC cells. All samples were centrifuged and re-suspended in cold binding buffer and PI (30.0 µg/ml). Detection of signals from a 2.0 × 10⁴ cells were obtained using the BD FACS Calibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA) and CellQuest Pro IVD software (Becton Dickinson, Mountain View, CA, USA).

DNA fragmentation assay

Cells were treated with AEA for 12 and 24 h before harvesting and total DNA was extracted from both untreated and treated cells using the Suicide Track™ DNA Ladder Isolation Kit according to the manufacturer’s protocol. Extracted DNA was analysed on a 1.0%/1.0 M of each compound.

Statistical analysis

All cell proliferation and flow cytometry experiments were carried out in triplicates. Data from all experiments were presented as mean values with ±standard deviation (SD). One-way ANOVA was used to determine the statistical significance of results with p < 0.05.

Results

Isolation and purification of phenylpropanoids from Alpinia conchigera

The main focus of this study was to chemically characterize AEA and other ACA natural analogs from Alpinia conchigera, followed by implicating their degree of cytotoxicity and apoptotic effects in the treatment of human breast cancer. Isolation and purification of the compounds from dichloromethane (CH₂Cl₂) extract yielded six purified compounds: 1′S-1′-acetoxychavicol acetate, 1 (1.62 g, 23.1%), 1′S-1′-acetoxyeugenol acetate, 2 (3.0 mg, 0.04%), 1′-hydroxychavicol acetate, 3 (77.7 mg, 1.11%), 1′-coumaryl diacetate, 4 (153.8 mg, 2.2%), p-hydroxycinnamyl acetate, 5 (70.6 mg, 0.81%) and p-hydroxycinnamaldehyde, 6 (77.7 mg, 1.11%). The chemical structures of all six phenylpropanoids were found to correspond with previous reported references and are illustrated in Fig. 1.

AEA inhibits the proliferation of MCF-7

The MTT cell viability assay was used to examine the effects of ACA analogues on the proliferation of MCF-7 and HMEC cells.
As shown in Table 1, only ACA and AEA out of the six analogs tested were successful in inhibiting the growth of MCF-7 cells with IC₅₀ values of 20.0 and 14.0 μM respectively. Cytotoxicity induced by both ACA and AEA were found to be dose and time dependent with 100% inhibition achieved after 36 h of treatment. The anti-proliferative effects of both ACA and AEA were found to be minimal when tested on HMEC normal human breast cells control. Solvent controls showed that the viability of cells were insignificantly affected (data not shown), indicating that cytotoxicity was not induced by the presence of DMSO, which has been shown to be cytotoxic at high concentrations (Violante et al. 2002). AEA also displayed comparable levels of cytotoxicity with paclitaxel which was used as a positive control drug. Our preliminary results support the potential development of AEA as an anti-proliferative drug. Because the cytotoxicity effects ACA have been previously reported, we focused our attention to AEA, where all consecutive experiments were carried out based on IC₅₀ values obtained from our present MTT data.

AEA induces apoptosis mediated cell death in MCF-7 cells

Lastly, we investigated whether AEA was inducing cell death via apoptosis or necrosis in MCF-7 cells using DNA fragmentation assay and double fluorescence staining of annexin V-FITC/PI flow cytometry assay. As shown in Fig. 3A, the population of cells indicated a shift from viable cells to early and late stage apoptosis, followed by secondary necrosis after 12 and 24 h AEA treatment. The percentage of both early and late apoptotic cells increased from 2.84% to 36.13% after 24 h AEA exposure (Fig. 3B). The occurrence of apoptosis mediated cell death was confirmed through the activation endonucleases-mediated nucleosome excision leading to the observation of DNA laddering of about 180–200 bp. Fig. 3C demonstrated partial and complete fragmentation of MCF-7 genomic DNA after 12 and 24 h of AEA treatment respectively, which represents one of the major hallmarks of apoptosis.

Discussion

Cells undergoing apoptosis can be observed via multiple hallmarks such as the appearance of a sub-diploid DNA peak during PI-based cell cycle analysis, the staining of exposed phosphatidylserine on the cell surface which is demonstrated during annexin V-FITC/PI flow cytometry, and the consistent laddering of genomic DNA (Renehan et al. 2001; Majno and Joris 1995). All observations reported in this study strongly suggest that AEA induces both anti-proliferative and apoptotic effects on MCF-7 with no cytotoxic effects on HMEC normal human breast cells. Variations in cell cycle distribution after treatment with AEA, indicated by changes in PI intensity of stained DNA suggest that AEA inhibits cell cycle progression effects at the G₀/G₁ phase while unaffected HMEC normal cells. The effect of AEA in terms of cell cycle arrest in this study has been consistent with past reports on ACA, where
it strongly increased the population of myeloma cells in the G0–G1 phase and the sub-G1 phase (Ito et al. 2005). Our current cell cycle studies in response to AEA treatments thus necessitates the need for additional molecular signalling studies on specific cyclin regulatory proteins, particularly CDK4 and CDK6 which are involved in G1 progression, and also various CDK inhibitors which may be up-regulated following AEA treatment.

Previous reports have shown that ACA from different plant species inhibits cellular carcinogenesis through various means such as the mitochondrial- and fas-mediated pathway (Ito et al. 2004), the inhibition of iNOS gene (Ohata et al. 1998), the induction of TRAIL (Ito et al. 2005) and the suppression of NF-kB pathway and its regulated gene products (Ichikawa et al. 2005). Since NF-kB acts as a transcription factor that regulates the transcription of genes corresponding to immune responses, proliferation, inflammation, anti-/pro-apoptotic genes and cell cycle regulators (Beinke and Ley 2004), it is likely that both ACA and AEA shares similar apoptotic mechanisms by primarily targeting the NF-kB pathway, which could then trigger a cascade of rippling effects on other downstream apoptotic pathways.

Considering that only ACA and AEA (out of all six phenylpropanoid compounds) were successful in manifesting its cytotoxic effects, a few observations were noted. On the basis of structure–activity relationship studies, the contributions of 2′,3′- terminal double bond of ACA and AEA is highly responsible for activity. Evidently, analogue 4, 5 and 6 which were devoid of the 2′-3′ terminal double bond did not show significant cytotoxic effect on MCF-7 cells. In addition, the para substitution of the acetoxy and 1′-acetoxypropenyl group at the benzene ring of ACA and AEA were found to be essential for biological activity. Murakami et al reported that both acetoxy group in ACA and AEA were necessary in cellular permeability properties because the analogue without the 1′-acetyl group (1′S,1′R-hydroxychavicol acetate, 3) resulted in the elimination of its cytotoxicity.

Previous studies using esterase inhibitors also found that the acetoxy groups in ACA were subjected to acetate elimination through hydrolyzation by intracellular esterase activity in order to maintain its retention within the cell (Murakami et al 2000) thus resulting in an intracellular modified ACA candidate structure which targets specific downstream molecules. To date, little has been known about the chemical properties of AEA in relation to its biological implications in comparison to ACA. We are currently in the process of elucidating the molecular mechanism by which AEA modulates its effects through global gene expression and proteins expression studies, as well as evaluating the synergistic effects of AEA as a chemosensitizer when used in combination with other anti-cancer drugs.

Overall, this study provides evidence of 1′S-1′-acetoxyeugenol acetate (AEA) isolated from the Malaysian Alpinia conchigera Griff., of possessing anti-cancer properties through the induction of cell cycle arrest and apoptosis in MCF-7 human breast carcinoma. Our current findings also conclude that AEA induces comparable, if not, better cytotoxic effects with paclitaxel, and is a significantly more potent anti-cancer compound in comparison to its analogue, ACA.

Acknowledgements

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