1′S-1′-acetoxyeugenol acetate: a novel phenylpropanoid from *Alpinia conchigera* enhances the apoptotic effects of paclitaxel in MCF-7 cells through NF-κB inactivation

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In this study, the apoptotic mechanism and combinatorial chemotherapeutic effects of the cytotoxic phenylpropanoid compound 1′S-1′-acetoxyeugenol acetate (AEA), extracted from rhizomes of the Malaysian ethnomedicinal plant *Alpinia conchigera* Griff. (Zingiberaceae), on MCF-7 human breast cancer cells were investigated for the first time. Data from cytotoxic and apoptotic assays such as live and dead and poly-(ADP-ribose) polymerase cleavage assays indicated that AEA was able to induce apoptosis in MCF-7 cells, but not in normal human mammary epithelial cells. A microarray global gene expression analysis of MCF-7 cells, treated with AEA, suggested that the induction of tumor cell death through apoptosis was modulated through dysregulation of the nuclear factor-kappaB (NF-κB) pathway, as shown by the reduced expression of various κB-regulated gene targets. Consequent to this, western blot analysis of proteins corresponding to the NF-κB pathway indicated that AEA inhibited phosphorylation levels of the inhibitor of κB–kinase complex, resulting in the elimination of apoptotic resistance originating from NF-κB activation. This AEA-based apoptotic modulation was elucidated for the first time in this study, and gave rise to the proposal of an NF-κB model termed the 'Switching/Alternating Model.' In addition to this, AEA was also found to synergistically enhance the proapoptotic effects of paclitaxel, when used in combination with MCF-7 cells, presumably by a chemosensitizing role. Therefore, it was concluded that AEA isolated from the Malaysian tropical ginger (*A. conchigera*) served as a very promising candidate for further in-vivo development in animal models and in subsequent clinical trials involving patients with breast-related malignancies. *Anti-Cancer Drugs* 22:424–434 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Worldwide, breast cancer is the second most common cancer among women after skin cancer, and it is also the second leading cause of cancer deaths in women after lung cancer [1]. In Malaysia, the National Cancer Registry reported that there were 3783 female breast cancer cases amounting to 31% of total cancer cases in 2003, making it the most commonly diagnosed type of cancer among women in Peninsular Malaysia. Hormone exposure, family genotypes, alcohol consumption, early menarche, late menopause, low parity, and postmenopausal obesity are all found to be established risk factors of breast cancer [2].

Traditional medicine has been a fertile source for revealing novel lead molecules for modern drug discovery, where various plants species have been used as medicines for thousands of years. *Alpinia conchigera* is a herbaceous perennial, 2–5 ft tall, found in eastern Bengal and southward toward Peninsular Malaysia and Sumatra. 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 gastrointestinal disorders and in the preparation of Thai food dishes [3]. In Malaysia, it is also locally known as *lengkuas ranting*, *lengkuas kecil*, *lengkuas padang*, *lengkuas geting*, or *chengkenam* [4].

Numerous natural compounds extracted from various plants have shown great promise in the therapeutic treatment of cancer and inflammatory diseases. Among these compounds is 1′S-1′-acetoxyeugenol acetate (AEA), which is a closely related phenylpropanoid analog of the well-studied 1′S-1′-acetoxychavicol acetate (ACA), found naturally in various plant species of the Zingiberaceae family [5]. The active chemical structure of AEA resembles that of ACA, with the exception of the former having an additional methoxyl group at the C-3 position of the benzene ring. The proapoptotic effect of ACA from the Thai ginger isolate *Languas galanga* has been

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documented earlier in human breast carcinoma cells [6], human T-cell lymphoma [7], and in the inhibition of tumor-promoter-induced Epstein–Barr virus [8]. Even though earlier studies have shown that ACA isolates from ginger exhibit antitumor properties against a wide variety of cancers, there has been no study so far on the cytotoxic and apoptotic effects of the closely related analog, AEA, on cancer cell lines.

Nuclear factor-kappaB (NF-κB) is a transcription factor, which is constitutively present in the cytoplasm as an inactive heterotrimer consisting of p50, p65 (RelA), and inhibitor of κB-α subunits. On activation by various cytokines and chemokines, IκBα undergoes inhibitor of κB kinase (IKK)-mediated phosphorylation and subsequent ubiquitination-dependent degradation, allowing NF-κB heterodimers to freely translocate into the nucleus to promote transcription of over 250 genes [9]. The expression of most NF-κB-regulated genes has been shown to be linked to most cancers, and can mediate events such as cellular transformation, proliferation, invasion, angiogenesis, and metastasis [10].

The constitutive expression of NF-κB activity has been documented in mammary carcinoma cell lines and in in-vivo primary breast cancers, regardless of their hormone-dependency cancer forms [11–13]. It has also been reported in preclinical studies that estrogen receptor-positive breast cancers can restore its sensitivity toward endocrine agents such as tamoxifen through the interruption of NF-κB activation [14]. In the past, there have been studies on various chemotherapeutic agents that were able to cause dysregulation of NF-κB and NF-κB-regulated gene targets, leading to apoptosis [15–17]. Therefore, in this study, apoptotic modulations in relation to NF-κB activation, as well as combinatorial chemotherapeutic effects of the cytotoxic phenylpropanoid compound AEA, extracted from the rhizomes of the Malaysian ethnomedicinal plant *A. conchigera* Griff. (Zingiberaceae), were investigated for the first time on MCF-7 human breast cancer cells.

Materials and methods

Plant material

Rhizomes of *A. conchigera* Griff. were collected from the Jeli province of Kelantan, east coast of Peninsular Malaysia. The sample was identified by Professor Dr Halijah Ibrahim (Institute of Biological Science, Faculty of Science, University of Malaya). A voucher specimen (KL5049) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University of Malaya. Extraction, purification, and characterization of active AEA compounds were carried out as described earlier [5].

Reagents

RPMI 1640, mammary epithelial growth media, fetal bovine serum, trypsin, and all antibiotics were purchased from Lonza Inc. (Allendale, New Jersey, USA). MTT reagent, bovine serum albumin, cisplatin, gemcitabine-HCl, and paclitaxel were obtained from EMD Chemicals Inc., (San Diego, California, USA). Primary total and phosphorylated NF-κB antibodies p50, p65, p52, IκBα, IκBβ, IKKα, IKKβ, NEMO, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Antibodies against poly-(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling (Danvers, Massachusetts, USA).

Cell lines and culture conditions

Two human cell lines were used in this study: human breast adenocarcinoma (MCF-7) cells, which were obtained from Dr Eswary Thirthagiri (Cancer Research Initiative Foundation, Malaysia) in 2009, and human mammary epithelial cells (HMEC) which were purchased in 2009 (Lonza), were used as normal cell controls. All cells were cultured as monolayers in RPMI 1640, supplemented with 10.0% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, whereas HMEC cells were cultured in mammary epithelial growth media. All running cultures were maintained at 10 passages or fewer in a humidified incubator at 37°C in 5% CO_2 and 95% air.

Live and dead cytotoxicity assay

Assessment of cell viability on treatment with AEA was accomplished using the LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian Cells (Molecular Probes, Invitrogen, USA) according to the manufacturer's protocol. Cancer and normal cell lines were grown as monolayers on coverslips for 24 h and treated with AEA for 3 and 6 h. Cells were stained using a dual fluorescence staining system consisting of 150 μl of both calcein acetoxymethyl ester (2 μmol/l; Molecular Probes), which emits green fluorescence when cleaved by intracellular esterases, and ethidium homodimer (4 μmol/l; Molecular Probes) which emits red fluorescence on binding to nucleic acid in nonviable cells. Excitation and emission wavelengths of both fluoresceins were set at 494/517 nm for calcein acetoxymethyl ester and 528/617 nm for ethidium homodimer. Samples were visualized using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Japan) under × 100 magnification with dual pass filters for simultaneous viewing of both stains.

Poly-(ADP-ribose) polymerase cleavage assay

Apoptosis was assessed by determining the proteolytic cleavage of PARP. In brief, cells (2×10^6/ml) were treated with AEA, and total proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, USA) according to the manufacturer's protocol. Fractionation was performed using SDS-PAGE and electrotransferred onto nitrocellulose membranes. Total proteins were incubated with rabbit anti-PARP antibodies and detected

using an enhanced chemiluminescence reagent (Pierce) using radiographic films. Apoptosis was represented by a cleavage of 116-kDa PARP into an 85-kDa product.

Microarray global gene expression analysis

To assay for changes in global gene expression before and after AEA treatment, the Affymetrix GeneChip Human Gene 1.0 Sense Target Array (Affymetrix Inc., USA) was used according to the manufacturer's protocol. In brief, total RNA from MCF-7 cells treated with AEA for 60 and 120 min was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol and analyzed under the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). RNA samples were then reverse transcribed, labeled, and hybridized onto Affymetrix chips containing 764 885 probes representing and spanning across 28 869 human genes. All arrays were scanned using the Affymetrix GeneChip Scanner (Affymetrix Inc.). Statistical and gene expression analyses of triplicate arrays were carried out using the GeneSpring GX version 10.0 (Agilent Technologies) software using principal component analysis plots and specific *P* value and fold-change thresholds.

Western blot analysis

To determine NF-κB levels of protein expression, AEA-treated whole-cell extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce) according to the manufacturer's protocol and fractionated using 12% (w/v) SDS-PAGE. After electrophoresis, proteins were electrotransferred using a TransBlot SD blotter (BioRad, USA) onto 0.2 μm nitrocellulose membranes, blocked with 2% (w/v) BSA and 0.5% (w/v) nonfat skim dry milk, and incubated with primary and secondary antibodies. Bound antibodies were detected with an enhanced chemiluminescence reagent (Pierce) using radiographic films. Normalization of protein concentrations and loading controls was carried out using the Bradford assay and rabbit anti-β-actin antibodies. Anti-tubulin and antihistone antibodies were used as cytoplasmic and nuclear component controls, respectively. Relative intensities of all bands were quantified using image analysis software (NIH ImageJ version 1.43u; National Institute of Health, USA).

In-vitro combination assays

Synergistic drug combination treatments between AEA and paclitaxel were evaluated using MTT assays on MCF-7 cells. A total of 2×10^4 cells were plated in triplicate and treated with either standalone AEA, standalone paclitaxel, or AEA in combination with paclitaxel at various concentration ratios for a duration of 24 and 48 h exposure. After incubation, 5 mg/ml MTT reagent (Calbiochem, USA) was added to each well and incubated for 2 h in the dark at 37°C until a purple formazan precipitate was clearly visible. Absorbance was measured at a wavelength of 570 nm with a reference wavelength of 650 nm using the Tecan Sunrise microtitre plate reader (Tecan, Switzerland). The type of combination relationship was assessed using an isobologram analysis, whereas the degree of synergy was assessed on the basis of calculated combination index (CI) values, where CI values of greater than 1.0 implies antagonism, 1.0 implies additivity, and a value of less than 1.0 implies synergy-type relationships between the two drugs. All calculations were based on the CI equation adapted from earlier literature [18].

Statistical analysis

All global gene expression and in-vitro drug combination experiments were carried out in triplicate. Data from all experiments were presented as mean values with ± standard deviation. One-way analysis of variance was used to determine the statistical significance of results with a *P* value of less than or equal to 0.05.

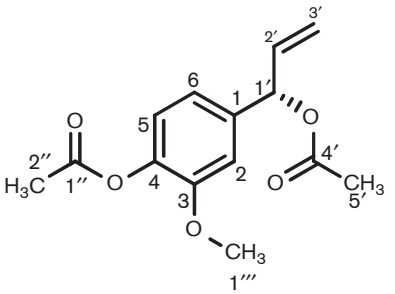
Results

The main focus of this study was to determine whether AEA (Fig. 1) plays a chemotherapeutic role in the prevention or treatment of human breast cancer. Subsequent to our initial studies on AEA cytotoxicity [5], we proceed to confirm the possible mechanisms of how AEA induces apoptosis in MCF-7 cells, through an overview of global gene expression changes, followed by focusing on specific pathways such as NF-κB, which has been shown to be affected by the closely related phenylpropanoid, ACA [7,19,20], and also because of its overwhelming involvement in the prevention and treatment of various types of cancer [10,21].

AEA induces time-dependent cytotoxicity and apoptosis in MCF-7 cells

We first examined the cytotoxic effect of AEA on the proliferation of MCF-7 and HMEC cells using the live and dead cell viability fluorescence assay. Treatment with AEA at 40 μmol/l concentrations was found to induce cytotoxicity in MCF-7 breast cancer cells in a time-dependent

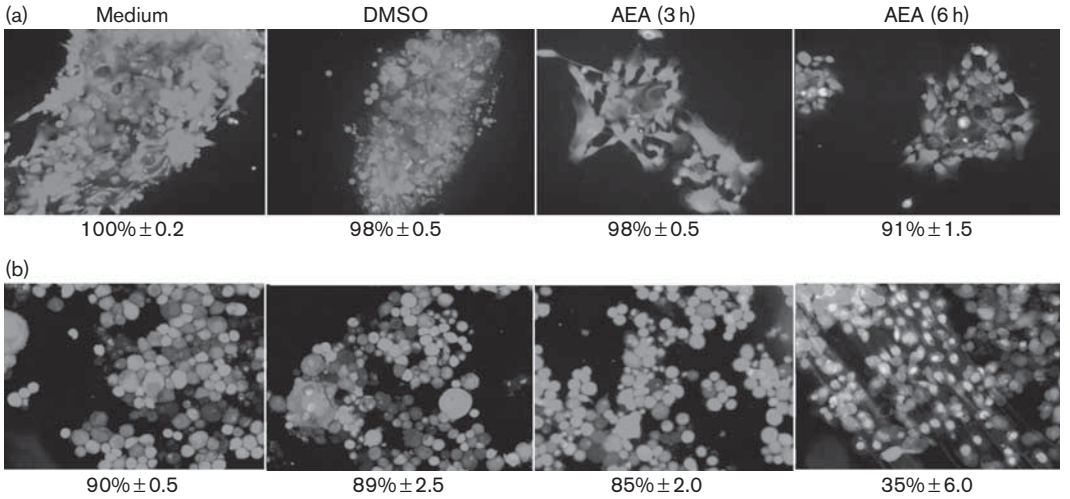
Fig. 1



1′S-1′-acetoxyeugenol acetate

Chemical structure of 1′S-1′-acetoxyeugenol acetate isolated from *Alpinia conchigera* (Zingiberaceae family).

Fig. 2



Live and dead viability/cytotoxicity assay depicting the cytotoxic effects of 1′S-1′-acetoxyeugenol acetate (AEA) in a time-dependent manner in MCF-7 cells (b), and with minimal effects on human mammary epithelial cells normal cell viability (a). Viable cells stained with acetomethoxy derivate of calcein, whereas nonviable cells stained with ethidium homodimer 1. Relative mean percentages of viable cells are shown as calculated under a fluorescence microscope, with a $P \leq 0.05$ of three independent experiments ($n=3$). A total of four random quadrants were selected from each replicate for quantification.

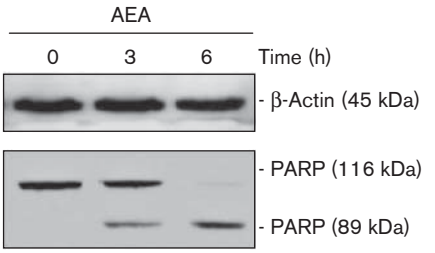
manner over 6 h of exposure, wherein cell viability level was reduced to 35% (Fig. 2b). Dimethyl sulfoxide solvent controls indicated minimal viability reductions between 2 and 11%, thereby ruling out any significant solvent-induced cytotoxicity in all cell lines tested. When tested on HMEC normal cell controls, AEA did not cause any significant cytotoxic effects, with the viability of HMEC cells maintained above 90% after 6 h of exposure (Fig. 2a). AEA concentration values used in this study were based on our initial report on AEA biological activity [5].

Thereafter, we investigated whether AEA mediated apoptosis in MCF-7 human breast cancer cells through the immunoblotting assessment of PARP cleavage. We found that treatment with AEA for 3 and 6 h induced caspase-3-mediated PARP cleavage in a time-dependent manner, consistent with increased levels of cleaved PARP of 89-kDa large fragments (Fig. 3). These results confirmed the occurrence of apoptosis-mediated cell death through caspase-3-dependent activation induced by AEA on human breast cancers *in vitro*, and was found to be consistent with earlier DNA fragmentation and flow cytometric apoptosis-confirmation assays in our initial study [5].

Global gene expression patterns indicate that AEA induces apoptosis by dysregulation of the NF-κB pathway

To investigate how AEA mediated apoptosis in breast cancer cells, the global gene expression profiles of AEA-treated MCF-7 cells were compared using the GeneChip Human Gene 1.0 Sense Target array consisting of approximately 764 885 probes spanning the full length

Fig. 3



1′S-1′-acetoxyeugenol acetate (AEA) mediates apoptosis-mediated cell death through activation of caspase-3 and subsequent cleavage of full-length poly-(ADP-ribose) polymerase (PARP) enzymes (116 kDa) into a small (24 kDa) subunit and a large (89 kDa) subunit protein. MCF-7 human breast cancer cells (2×10^6) were treated with 40 μmol/l AEA for 3 and 6 h. Whole-cell extracts were prepared and subjected to western blot analysis using anti-PARP antibodies. β-Actin was used as a normalization control to ensure equal protein concentrations across samples.

of approximately 28 869 genes within the entire human genome. Normalization and filtering of raw microarray data, based on *P* values and significant fold-changes, were conducted resulting in a finalized list consisting of 123 genes. The top 20 upregulated and downregulated genes related to the apoptosis and carcinogenicity are compiled in relation to the NF-κB pathway and summarized in Table 1.

Among the top upregulated genes, on AEA treatment, were F-box proteins, ubiquitin-specific peptidases, and small mother against decapentaplegic-specific E3 ubiquitin protein ligases, all of which played significant

roles in the ubiquitination of proteins targeted for proteosomal degradation, such as IκBs. It was also interesting to note that some of the Ras superfamily members, such as the NF-κB inhibitor-interacting Ras-like 1 and 2 genes, which are often mutated in a majority of cancer types (Bos [22]), were found to be upregulated during AEA treatment in MCF-7 cells. Other MCF-7 genes that were also found to be upregulated after AEA exposure were those encoding CDH2, which are known NF-κB inhibitors in melanoma cells [23], and *TGFBR2*, *IGF1R*, and *IFNA1*, all of which are gene targets of NF-κB, containing the κB consensus sequence (Table 1).

In terms of genes downregulated upon AEA treatment, it was observed that multiple members of the tumor necrosis factor (TNF) superfamily, such as the lymphotoxin α (TNF superfamily, member 1) gene, TNFR (TNF superfamily, member 4 and 8) genes, and the TNF-α-induced protein 1 gene, were all affected. All these TNF superfamily genes were found to contain the κB sequence site within its promoter region, indicating their correlation toward NF-κB inactivation. Other downregulated genes affected by AEA include both NF-κB activators and coactivators such as *MDM4*, *BCORL2*, *PARP10*, *FGFR4*, and *MAPK13* (Table 1).

AEA downregulates nuclear factor-kappaB activation in MCF-7 cells

Western blot analysis was used to investigate whether proapoptotic effects of AEA are mediated through modulation of the NF-κB regulatory pathway, which is known to cause chemoresistance, inflammatory, and antiapoptotic effects. Analysis of NF-κB heterodimer localization levels within the nucleus and cytoplasm through western blotting assays was carried out using antibodies against p50. This protein was chosen because p50/RelA complexes have been shown to be the most common and dominant NF-κB heterodimer form in most cancer types [24,25]. It was observed that protein levels of p50 within the cytoplasm increase corresponding to increasing AEA exposure time (Fig. 4a). This was consistent with a decrease in nuclear p50 levels, indicating that p50/RelA heterodimers were being shuttled out from the nucleus at a faster rate compared with its translocation rate into the nucleus (Fig. 4a).

Consequent to observations indicating reduced p50 levels within the nucleus, the presence of activated NF-κB heterodimers was also assessed on the basis of phosphorylation levels of p65/p50 and p65/p52 heterodimers. It was found that AEA reduced p65 and p50 subunit phosphorylation in MCF-7 cells treated with AEA (Fig. 4b), suggesting that AEA prevented the C-terminal Ser 536 and Ser 337 phosphorylation, respectively, required for transactivation and commencement of κB promoter gene transcription activity. Despite reductions in p50 phosphorylation levels, it was interesting to note that p52 phosphorylation levels at Ser 865 increased upon AEA treatment in MCF-7 cells (Fig. 4b), suggesting that AEA may somehow play a role in the induction of MCF-7 apoptosis through the noncanonical pathway. Alternatively, p52 activation in MCF-7 cells could be explained by its application as a p52/p52 homodimer-based inhibitor, creating a competitive inhibitory mechanism for p65 subunits.

AEA inhibits TNF-α-induced MCF-7 cells through IKKα inactivation

In addition, we also investigated the ability of AEA to quench TNF-α-based activation of NF-κB in MCF-7 cells.

Table 1 Summary of the top 10 upregulated and downregulated cancer and apoptosis-related gene expression changes in MCF-7 cells after 1′S-1′-acetoxyeugenol acetate treatment for 1 and 2 h

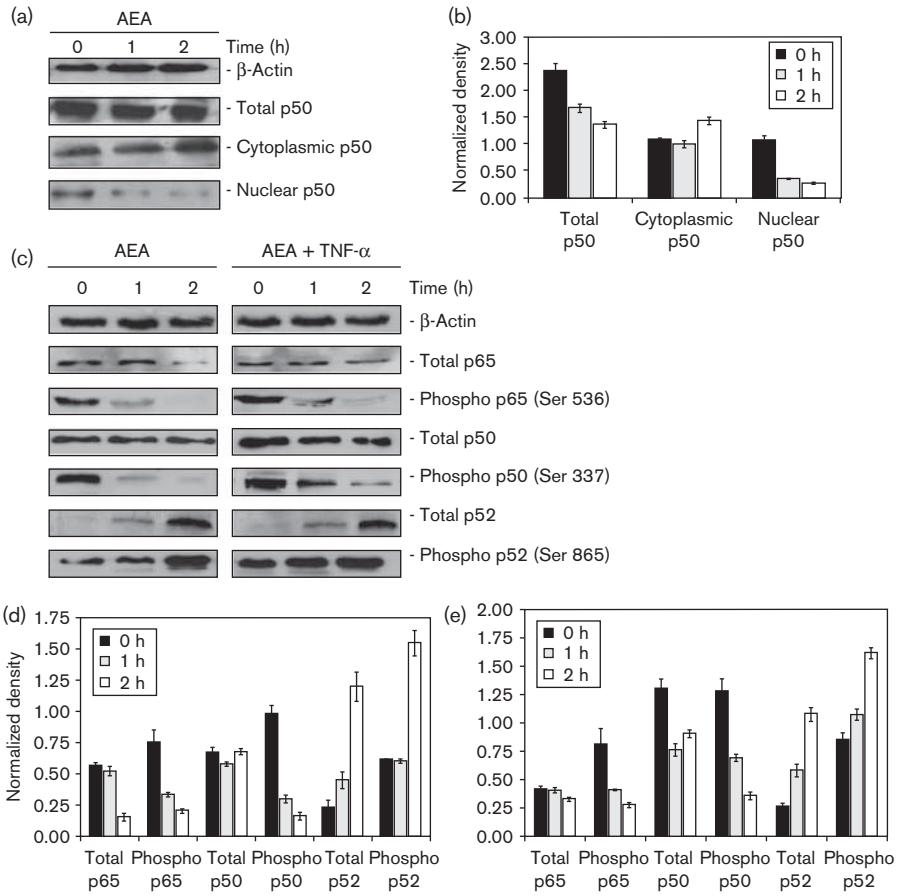
Gene description	GenBank ID	*P* value	Fold change (0 vs. 1)	Fold change (1 vs. 2)	NF-κB relation
F-box protein 11	BC130445	0.0283	2.30	1.92	Ubq.[a]
SMAD specific E3 ubiquitin protein ligase 2	AY014180	0.0367	1.60	1.57	Ubq.
N-cadherin 2	BC036470	0.0279	1.57	1.56	Inhibitor
TP53RK binding protein	BC029492	0.0045	1.56	1.52	Activator
Ubiquitin specific peptidase 29	BC130394	0.0315	1.54	1.51	Ubq.
Transforming growth factor, β receptor 2	M85079	0.0299	1.53	1.50	Gene target[b]
Insulin-like growth factor 1 receptor	BC010607	0.0054	1.53	1.55	Gene target
NF-κB inhibitor interacting Ras-like 2	BC063498	0.0064	1.52	1.59	Inhibitor
Interferon, α 2	M54886	0.0322	1.51	1.51	Gene target
NF-κB inhibitor interacting Ras-like 1	BC012145	0.0099	1.50	1.54	Inhibitor
Lymphotoxin α (TNF superfamily, member 1)	D12614	0.0462	−2.21	−2.16	Gene target
TNF receptor superfamily, member 4	BC105070	0.0237	−1.62	−1.54	Gene target
Proteasome (macropain) 26S subunit, nonATPase, 8	BC001164	0.0494	−1.61	−1.67	Gene target
TNF receptor superfamily, member 8	M83554	0.0346	−1.58	−1.63	Gene target
Mdm4 p53 binding protein homolog	BC067299	0.0061	−1.56	−1.51	Indirect activation[c]
BCL6 corepressor-like 2	BC063452	0.0037	−1.54	−1.56	Indirect activation
Poly (ADP-ribose) polymerase family, member 10	BC132961	0.0056	−1.54	−1.60	Activator
Tumour necrosis factor, α-induced protein 1	M80783	0.0186	−1.53	−1.51	Gene target
Fibroblast growth factor receptor 4	BC011847	0.0248	−1.53	−1.55	Activator
Mitogen-activated protein kinase 13	BC001641	0.0070	−1.52	−1.51	Activator

Genes were selected based on triplicates samples with $P < 0.05$ and mean fold changes > 1.50.
NF-κB, nuclear factor-kappaB; SMAD, small mother against decapentaplegic; TNF, tumor necrosis factor.
[a]Ubq.: involved in ubiquitination process.
[b]Gene target: contains κB site in promoter region.
[c]Indirect activation: gene is activated by another κB-regulated gene.

Western blot assays indicated that AEA was able to diminish TNF-α-based NF-κB activation through reductions in p65 and p50 phosphorylation levels in MCF-7 cells (Fig. 4b), further suggesting that AEA could hypothetically induce apoptosis in various TNF-α-transformed malignancies in addition to MCF-7 breast adenocarcinoma. However, whether the induction of apoptosis by AEA was achieved through inhibition of TNF-receptor-associated death domain-based proliferation or a redirection to TNF-receptor-associated death domain-based apoptotic mechanisms remains unknown. Assessment of upstream NF-κB member proteins was also made, and it was found that Ser 32/36 phosphorylation levels of IκBα proteins were reduced at 2 h of AEA exposure (Fig. 5a). This led to the suggestion that AEA prevented the degradation of IκBα proteins by preventing IKK-based phosphorylation and subsequent ubiquitination signaling. Similar dephosphorylation patterns in IκBα proteins were also observed in TNF-α-stimulated MCF-7 cells (Fig. 5a).

Western blot assays performed on both total and phosphorylated IKKα and IKKβ proteins after AEA treatment showed that levels of phosphorylated IKKα on Thr 23 and IKKβ on Ser 176 were reduced consistently with increasing AEA incubation periods (Fig. 5b). These observations suggested that reductions in IKKβ activation would prevent the interaction with at least three other kinase proteins such as mitogen-activated protein kinase 3, MAPK/ERK kinase 3, and transforming growth-factor-β-activated kinase 1, which are known to be responsible for IκBα degradation [26,27]. A reduction in IKKα phosphorylation also suggested that the noncanonical pathway was somewhat affected as IKKα is required for NF-κB inducing kinase-based processing of p100 into p52 [28,29]. Pretreatment of

Fig. 4



1′S-1′-acetoxyeugenol acetate (AEA) downregulates nuclear factor-κB (NF-κB) activation in MCF-7 breast cancer cells. Cancer cells (2×10^6) were treated with either AEA (40 μmol/l) or pretreated with tumor necrosis factor α (TNF-α) (10.0 ng/ml) for 1 and 2 h. Cytoplasmic and nuclear extracts were prepared and subjected to western blot analysis. Antitubulin and antihistone antibodies were used as cytoplasmic and nuclear component controls, respectively. (a) Western blot analysis of p50 (NF-κB1) protein localization levels between the cytoplasmic and nuclear portions upon treatment with AEA. (b) Normalized quantification of p50 localization. (c) Western blot analysis on various NF-κB protein member activation consisting of p65, p50, and p52 phosphorylation levels on AEA treatment and in combination with TNF-α pretreatment. (d) Normalized quantification of NF-κB protein members on AEA treatment. (e) Normalized quantification of NF-κB protein members on TNF-α stimulation followed by AEA treatment.

MCF-7 cells with TNF-α, which is known to activate IKKβ, was also found to be countered by the presence of AEA (Fig. 5b).

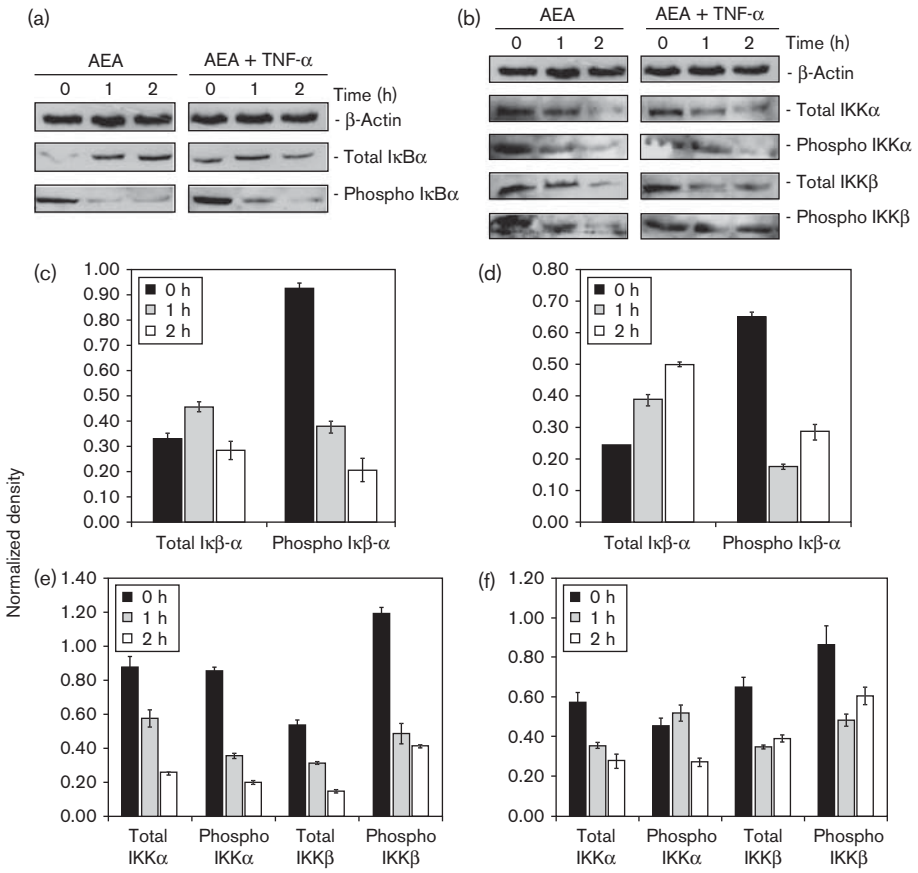
AEA enhances the apoptotic effects of paclitaxel through chemosensitization

As AEA was found to induce cytotoxicity in MCF-7 cells, we next sought to determine whether AEA could enhance the apoptotic effects of the chemotherapeutic drug, paclitaxel, when used in combination against breast cancer cells. On the basis of MTT assay isobologram-illustrated results, it was found that AEA was able to reduce the viability levels of MCF-7 cells to a higher extent when used in combination with paclitaxel (Fig. 6a). CI analysis indicated that synergistic effects were observed for all combinations of paclitaxel and AEA for 24 h (CI = 0.51–0.66). However, a shift toward an antagonistic effect was observed when treatment of MCF-7 cells was allowed to proceed for 48 h (CI = 1.02–1.06) (Fig. 6b). This indicated that AEA was necessary to sensitize MCF-7 cells toward apoptosis, but this sensitization effect was found to have a transient effect, diminishing upon 48 h of incubation. Sequential 12 h pre-AEA treatment before paclitaxel administration also showed a similar shift from a synergistic to an antagonistic relationship for both 24 and 48 h paclitaxel treatments, thus confirming the transient sensitization effect of AEA on MCF-7 cells (Fig. 6b).

Discussion

The overall purpose of this study was to investigate whether the natural compound AEA has a possible role in the treatment and/or prevention of breast cancer patients through the evaluation of affected pathways and its drug combination properties. Various types of chemosensitizers can and have been shown to sensitize cancer cells through various ways. For example, rituximab, a chimeric anti-CD20 monoclonal antibody, is able to interfere with the signal transduction pathway in nonHodgkin's lymphoma B cells

Fig. 5



1′S-1′-acetoxyeugenol acetate (AEA) inhibits the inhibitor of κB kinase α (IKKα) and IKKβ activation resulting in the prevention of IκBα phosphorylation and subsequent degradation. (a) Western blot analysis of IκBα protein phosphorylation levels after AEA treatment on tumor necrosis factor α (TNF-α) (±) MCF-7 cells. (b) Normalized quantification of total and phospho-IκBα. (c) Normalized quantification of total and phospho-IκBα in TNF-α-induced MCF-7 cells. (d) Western blot analysis of IKKα and IKKβ protein phosphorylation levels after AEA treatment on TNF-α (±) MCF-7 cells. (e) Normalized quantification of IKKα and IKKβ protein phosphorylation. (f) Normalized quantification of IKKα and IKKβ protein phosphorylation in TNF-α-induced MCF-7 cells.

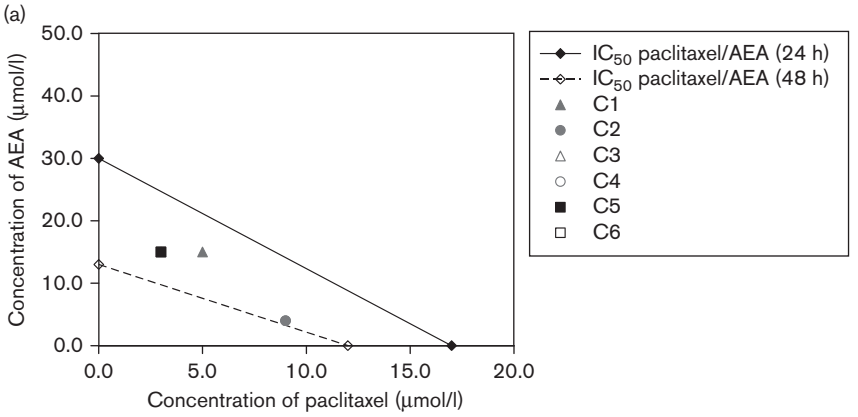
by selective downregulation of Bcl-2 and Bcl-xL [30]. As shown by earlier studies, multitargeted therapy has a higher success rate against cancer compared with mono-targeted therapies [31,32]. This study has also consistently shown that AEA isolated from *A. conchigera* was able to act as a chemosensitizing agent through the synergistic enhancement of apoptotic effects incurred by paclitaxel. Our findings also suggest that AEA can sensitize human breast cancer cells through the downregulation of NF-κB and NF-κB-modulated genes as shown by western blot results and microarray gene expression profiles.

NF-κB is known as an important and vital transcription factor because of its ability to protect cells from apoptosis through the exertion of various antiapoptotic genes [7,33,34]. Several facts have been presented implicating NF-κB in the oncogenesis of a wide array of malignancies such as head and neck, lymphoid, breast, gastric, colorectal, liver, cervical, and prostate cancers [35]. This further led to the implication of NF-κB activation as a major culprit in cancer pathogenesis, and also as a pathway of considerable importance in cancer. In this study, evidence revealed by microarray global gene expression data on AEA-treated MCF-7 cells has pointed out that NF-κB inactivation acted as the key control panel, which led to a series of apoptosis-inducing events. Microarray observations on various oncogenes that remain activated in the event of apoptosis are thought to be counterbalanced by the diminishing expression of other antiapoptotic genes, as well as by the induction of other tumor-suppressor genes, leading to conditions favoring apoptosis. Even though earlier literature has indicated the involvement of ACA, a close phenylpropanoid analog of AEA in NF-κB inactivation [7,19], it was nevertheless important to prove that AEA was also committed toward a similar mode of action.

Western blot data in this study clearly indicated the antiphosphorylation influence of AEA on key members of the NF-κB pathway. This consequently led to the proposal

Fig. 6

(a)



(b)

Drug	MW	Treatment (h)	IC_{50} (µg/ml)	IC_{50} (µmol/l)	CI value	Relationship
AEA	265.0	24	8.0	30.0	NA	NA
		48	3.4	13.0	NA	NA
Paclitaxel	853.9	24	14.5	17.0	NA	NA
		48	10.2	12.0	NA	NA
AEA + Paclitaxel	AEA constant	24	4.3	5.0	0.63 ± 0.05	Synergistic
		48	2.6	3.0	1.02 ± 0.12	Antagonistic
Paclitaxel + AEA	Paclitaxel constant	24	1.1	4.0	0.66 ± 0.08	Synergistic
		48	1.1	4.0	1.06 ± 0.11	Antagonistic
Pre-AEA + Paclitaxel	AEA constant	24	2.6	3.0	0.51 ± 0.08	Synergistic
		48	2.6	3.0	1.02 ± 0.06	Antagonistic

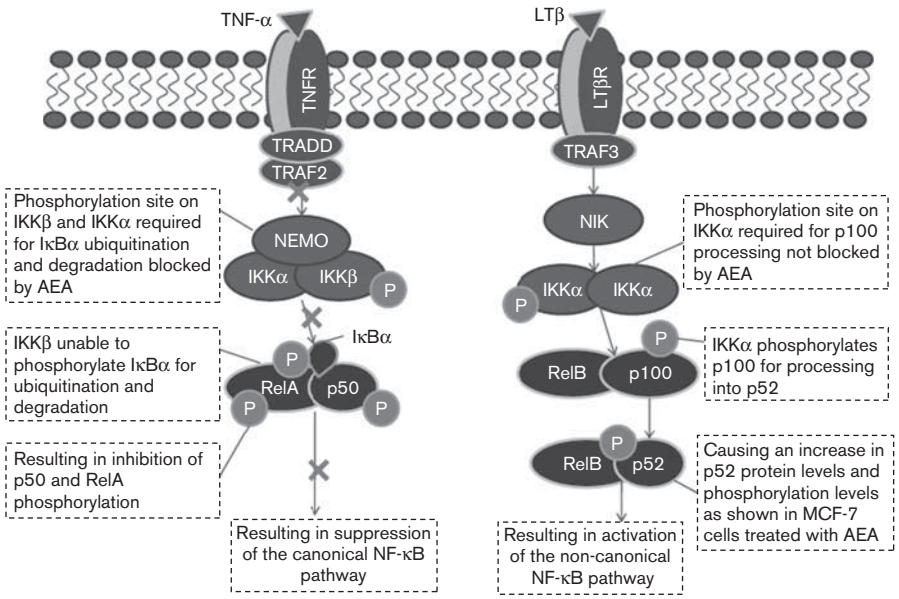
Observation of transient synergistic relationship between 1′S-1′-acetoxyeugenol acetate (AEA) and paclitaxel combination treatment suggesting that AEA plays a role in MCF-7 chemosensitization. (a) Isobologram IC_{50} analysis of MCF-7 cells after exposure to paclitaxel and AEA simultaneous and sequential combination treatment over 48 h. C1: 24 h simultaneous treatment with constant AEA; C2: 24 h simultaneous treatment with constant paclitaxel; C3: 48 h simultaneous treatment with constant AEA; C4: 48 h simultaneous treatment with constant paclitaxel; C5: 12 h pre-AEA treatment followed by 24 h of paclitaxel sequential treatment; C6: 12 h pre-AEA treatment followed by 48 h of paclitaxel sequential treatment. (b) Summary of IC_{50} and combination index (CI) values calculated from MTT cytotoxicity assays after various in-vitro combination treatments with AEA and paclitaxel on MCF-7 cells. Data are presented as mean values ± standard deviation of independent triplicate experiments.

of a molecular model describing the mode of action of AEA, termed the 'Switching/Alternating' Model (Fig. 7). In this model, it was suggested that AEA interacted with phosphorylation sites on both IKKβ and IKKα that were responsible for IκBα signalling (Thr 23 and Ser 176), but did not interact with phosphorylation sites on IKKα homodimer complexes that were required for p100 processing. Blockage of specific phosphorylation sites on IKKβ and IKKα thus prevented the ubiquitination and degradation of IκBα from the RelA/p50 heterodimer, resulting in the prevention of RelA and p50 phosphorylation. This resulted in the inhibition of the canonical mode of NF-κB activation, but did not affect the noncanonical mode of NF-κB activation as described by a rapid increase in p52 phosphorylation and protein levels upon AEA exposure in MCF-7 cells. Stipulations that different sites on the IKKα/IKKα complex were phosphorylated by different kinases such as NF-κB inducing kinase, mitogen-activated protein kinase 3, MAPK/ERK kinase, and transforming growth-factor-β-activated kinase 1, which in turn gave rise to different enzymatic target functions that served as a basis toward this model.

Apart from the apoptotic-inducing effects of AEA on conventional MCF-7 cells, we also showed the effectiveness of this compound on cancer cells subjected to TNF-α- enhanced NF-κB activation. TNF-α, a well-studied cytokine, is known to activate NF-κB and is one of the many transforming agents in tumorigenesis. In myeloma cell proliferation, TNF-α-based activation of NF-κB was linked with the upregulation of antiapoptotic proteins such as X-linked inhibitor of apoptosis protein and Fas-associated protein with death domain-like interleukin-1 β-converting enzyme-inhibitory protein, resulting in tumorigenesis [36]. Interestingly, the expression of cytokines such as interleukin-6 and TNF-α is also governed by NF-κB activation, creating a positive feedback regulatory loop [37]. Therefore, a simple activation of NF-κB can easily amplify its own activation by increasing the levels of TNF-α, further leading to the expression in other cytokines, chemokines, adhesion molecules, and enzymes present in tumorigenic cells. As a result of this reasoning, the effects of AEA were also assessed on cancer cell proliferation induced by TNF-α. As AEA was found to be effective against TNF-α-induced MCF-7 cells, its implications on other malignancies with increased TNF-α expression are therefore viable.

The discovery of new drugs that directly or indirectly affect the NF-κB pathway has also been implicated in diseases other than oncogenesis. The pathogeneses of certain inflammatory disorders such as asthma, rheumatoid arthritis [38], atherosclerosis [39], Alzheimer's disease [40], ulcerative colitis, and Crohn's disease [41] have all been shown to be linked with the NF-κB pathway. Even though the constitutive activation of NF-κB is mostly involved in the enhanced growth properties of a wide variety of cancers and inflammatory diseases, the complete blockage of this pathway may not be feasible as it induces severe side effects. In normal cells, although NF-κB is rarely constitutively expressed, with the exception of proliferating B cells, T cells, thymocytes, monocytes, and astrocytes, basal levels of NF-κB expression still remain

Fig. 7



A proposed 'Switching/Alternating Model' depicting 1′S-1′-acetoxyeugenol acetate (AEA) mode of action on the canonical and noncanonical nuclear factor-κB (NF-κB) pathway based on western blot results in MCF-7 cancer cells. IKKα, inhibitor of κB kinase α; TNF, tumor necrosis factor.

detectable [10]. The complete inhibition of NF-κB for prolonged periods may also jeopardize host defense and immune responses, rendering the host susceptible to diseases. Therefore, to better understand the mode of action of potential therapeutic agents such as AEA, and to identify further therapeutic strategies to regulate NF-κB-dependent immune responses, quantitative dynamic pharmacokinetic models of relevant signaling networks will be crucial.

This study was the first to show that natural AEA from the Malaysian wild ginger, *A. conchigera*, can suppress the activation of NF-κB and its regulated gene targets through the dephosphorylation of its subunits in human breast cancer cells. Newly emerging forms of combinatory chemotherapy involving potential chemosensitizers such as AEA and anticancer drugs have been gaining vast popularity among oncologists worldwide, especially in the field of personalized medicine [42]. The added understanding of how AEA potentiates apoptosis-mediated cell death is crucial in further understanding the mechanisms underlying carcinogenesis, hence giving cancer researchers a better insight into future drug development approaches. On the whole, our results support the use of AEA either alone or in combination with paclitaxel and perhaps with other existing therapies against MCF-7 breast cancer cells.

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