In Vitro Antimetastatic Activity of Koetjapic Acid against Breast Cancer Cells

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Received October 5, 2011; accepted December 12, 2011; published online January 23, 2012

Breast cancer is the most common cancer in women, and it can metastasize very rapidly. Tumor metastasis is the primary cause of cancer deaths. In the present study, we investigated the capability of koetjapic acid, a natural triterpene, in the induction of apoptosis and the inhibition of metastasis in the breast cancer cell line (MCF 7). The effects of koetjapic acid against 4 steps of metastasis have been assessed, including cell survival, clonogenicity, migration and invasion. Koetjapic acid exhibited cytotoxic activity against MCF 7 cells with an IC₅₀ of 68.88±6.075μg/mL. The mechanism of cell death was confirmed due to the induction of apoptosis machineries; early and late apoptosis-related changes were detected, including the stimulation of caspase 3/7 activities, apoptosis-related morphological changes such as membrane blebbing, chromat condensation and DNA fragmentation. A mitochondrial apoptosis pathway was found to be involved in koetjapic acid-induced cell death induction. Moreover, at a sub-toxic dose (15μg/mL), Koetjapic acid inhibited cell migration and invasion significantly. Finally, koetjapic acid inhibited the colony formation properties of MCF 7 significantly. These results indicate that koetjapic acid possesses significant antitumor and antimetastatic effects, and warrants further investigation.

Key words metastasis; breast cancer; natural product; migration; clonogenicity; apoptosis.

Cancer is a group of more than 100 different diseases characterized by uncontrolled cellular growth, local tissue invasion and distant metastasis. Breast cancer is the most common cancer in women; approximately 27% of all new cancers in women are breast cancer. Metastasis, the spread of a primary tumor from its initial location to distant localities, is the main cause of death in cancer patients. Metastatic cancers develop within 3 years after the initial detection of the primary tumor in almost 10–15% of patients.

The use of plants for medicinal purposes dates back to ancient times. The World Health Organization (WHO) estimates that 80% of the world’s population depends on natural sources for primary medical treatment, and natural sources are also incorporated in the medical treatment of the remaining 20%. However, out of the thousands of traditionally used natural products, it is estimated that only 5–15% have been scientifically proven.

Nowadays, cancer treatment protocols are mainly based on phytochemicals such as paclitaxel, vincristine and camptothecin. In general, the anticancer activity of these compounds is mediated via blocking the cell cycle, and via the induction of the apoptotic machineries of cell death. Hence, instigation of the programmed cell death in tumor cells has been considered the foremost indicator of the antitumor activity of naturally derived compounds.

Sandoricum koetjape (Meliaceae) is an evergreen tree. Traditionally, a decoction of bark has been used to heal leucorrhoea and colic, and it has also been used as a tonic after giving birth. In a previous report, koetjapic acid (KA) was purified from the n-hexane extract of S. koetjape bark (Fig. 1A). The compound has demonstrated interesting pharmacological effects including antiangiogenic, ichthyotoxic, antibacterial, and chemopreventive effects.

Since the n-hexane extract showed potential anti-proliferative activity against a breast cancer cell line, it has been suggested that KA may have potential value as an anti-breast cancer agent. Accordingly, this study has been performed in order to examine the growth inhibitory effect of KA on human breast cancer cells (MCF 7), and to elucidate the mechanism(s) involved in the action of KA. In addition, based on the compound’s antiangiogenic and chemopreventive properties, it is expected to show an antimetastatic effect. Hence, the effect of KA on key steps of tumor metastasis, including cell migration, invasion and clonogenicity, was also investigated.

MATERIALS AND METHODS

Chemicals and Reagents Modified Eagle’s medium (MEM), trypsin and heat inactivated fetal bovine serum (HIFBS) were obtained from Gibco, U.K. Matrigel matrix (10mg/mL) was supplied by BD Bioscience, U.S.A. Tamoxifen (TMX), phosphate-buffered saline (PBS), penicillin/streptomycin (PS) solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, crystal violet, rhodamine 123, and Hoechst 33258 were purchased from Sigma-Aldrich, U.S.A. KA was isolated from S. koetjape stem bark as described previously.

Cell lines and Culture Conditions Human breast cancer cell line (MCF 7, ATCC® HTB-22™) was purchased from ATCC (Rockville, MD, U.S.A.). The cells were cultured in MEM supplemented with 10% HIFBS and 1% PS. Cells were cultured in a 5% CO₂ in a humidified atmosphere at 37°C.

Cell Viability Assay Viability of the MCF 7 cells was determined by the MTT test as described previously. Briefly,
cells were seeded at $5 \times 10^3$ per well in 100 μL MEM medium, and incubated overnight to allow attachment. Cells were then exposed to varying concentrations of KA or TMX for 48 h. After incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added and incubated for an additional 3 h. Subsequently, the medium was aspirated carefully, and 150 μL dimethyl sulfoxide (DMSO) was added. After incubation, 20 μL of MTT solution was added and incubated for an additional 3 h. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min, washed with PBS, and stained simultaneously (30 min) with Hoechst 33258 at 10 μg/mL and rhodamine 123 at 5 μg/mL. Then, the cells were washed twice and observed under fluorescent microscopy (Olympus, Japan). For chromatin condensation analysis, cells with bright condensed or fragmented nuclei were considered apoptotic; the images were acquired after 6, 12 and 18 h. The loss of mitochondrial membrane potential was indicated by the appearance of more brightly-stained cells; the images were captured after 6, 9 and 12 h. The apoptotic cells were counted in 4 randomly selected fields per well. The apoptotic index was calculated as the percentage of apoptotic cells compared to the total number of cells, and was presented as a mean ± S.D. ($n=3$).

**DNA Fragmentation Assay** MCF 7 cells ($5 \times 10^5$/T-25 flask) were treated with 60 or 80 μg/mL of KA, 10 μg/mL TMX or 1% ethanol for 36 h. The floating and attached cells were collected by centrifugation, washed with PBS, and the DNA was purified using Wizard® SV Genomic DNA Purification Kit (Promega). DNA was then analyzed by electrophoresis on 1.2% agarose gel stained with ethidium bromide.

**Cell Migration Assay** The effect of KA on the migration of MCF 7 cells was studied by the wound healing assay as described previously.[17] MCF 7 cells were cultured in 6-well plates ($1 \times 10^6$ cells/well) in a medium containing 10% FBS. After the cells grew to confluence, wounds were made by sterile pipette tips. The cells were washed with PBS and incubated in serum-reduced medium (2% FBS) containing 15 μg/mL of KA. Control cells received the vehicle alone (1% ethanol). After 6, 9, 12 and 15 h incubation, the cells were fixed and photographed. The width of the cell-free wounds was measured using an inverted light microscope supplied with a Leica Quin computerized imaging system. Ten fields per well were photographed, and a minimum of 30 readings per field were taken. The results are presented as a percentage of wound closure ± S.D. ($n=3$).

**Cell Invasion Assay** The anti-invasion potential of KA was assessed as described previously.[18] In brief, 96-well plates were coated with 75 μL/well Matrigel (BD Bioscience) diluted in a culture medium at 5 μg/mL. Then, untreated or 15 μg/mL KA-treated tumor cells ($5 \times 10^5$) were added to each well. After 12 h incubation, the upper surface of the matrigel was wiped with a cotton-tipped applicator to remove non-migrated cells. Cells that invaded the matrigel layer were photographed (10× magnification). All experiments were run in triplicate. The results are presented as the average percentage of invasion inhibition relative to the negative control (1% ethanol).

**Clonogenicity Assay** The effect of KA on MCF 7 colony formation was evaluated as described previously.[19] MCF 7 cells in log phase growth were prepared in a single-cell suspension before being plated in a 6-well plate (500 cells/well). The cells were incubated for 24 h to facilitate their attachment, and treated with various concentrations of KA, TMX ($12.5 \mu g$/
April 2012 505

mL) as a positive control or 1% ethanol as a negative control. Following an additional 48 h incubation, treatments were removed, and cells were incubated in a drug-free medium for an additional 7 d. Colonies were then fixed with 4% paraformaldehyde for 30 min and stained with 0.2% (w/v) crystal violet for 20 min. The colonies with >50 cells were counted under a stereomicroscope and the plating efficiency (PE) and survival fraction (SF) were calculated. Results are expressed as the mean±S.D. (n=3).

Statistical Analysis Results are presented as means±S.D., and differences between groups were compared by One-way analysis of variance (ANOVA) and considered significant at p<0.05. The statistical analysis was carried out using SSPS edition 16.0.

RESULTS

KA Inhibited MCF 7 Proliferation The MTT cell proliferation assay was performed to study the effect of KA on MCF 7 cell viability. As shown in Fig. 1B, KA inhibited the proliferation of MCF 7 in a dose dependent manner. Although no significant effect on cell viability was detected at lower concentrations (10 and 20 μg/mL), KA at higher concentrations decreased the cell viability significantly with a median inhibitory concentration (IC50) of 68.88±6.075 μg/mL. TMX, a standard therapeutic drug for breast cancer, was used as a positive control, and showed significant toxicity towards MCF 7 cells with an IC50 of 9.41±0.33 μg/mL (Fig. 1C).

KA Activated Caspase 3/7 in MCF 7 Cells In this study, the effect of KA on caspase 3/7 activation was analyzed. Compared to untreated cells, treatment with KA at 25, 50

Fig. 2. (A) Effect of KA on Caspases 3/7 Activities (B) Effects of KA on DNA Fragmentation in MCF 7 Cells

(A) Fold induction was calculated by dividing the values obtained from KA-treated samples by the readings obtained from the control treated samples (1% ethanol). Cells were treated for 4 h. Values represent means of 3 experiments±S.D. *p<0.05. (B) Shown is a representative electrophoretic fragmentation pattern of DNA isolated from cells treated with (1) 60 μg/mL, (2) 80 μg/mL, (3) TMX 12.5 μg/mL and (4) the vehicle, 1% ethanol, for 48 h. The experiment was performed twice.

Fig. 3. Effect of KA on Nuclear Morphology of MCF 7 Cells

Images show Hoechst 33258-stained nuclei after treatment with 1% ethanol or indicated concentrations of KA for the indicated time. Typical results from 3 independent experiments were obtained. Cells demonstrated distinctive apoptosis-associated morphological changes: cell shrinkage, plasma and nuclear membrane blebbing, organelle re-localization and compaction and formation of apoptotic bodies. (C) KA effect on mitochondrial membrane potential and rhodamine 123 uptake after exposing the MCF 7 cells to 1% ethanol or indicated concentration and time. (B) The percent of apoptotic indexes calculated based on chromatin condensation and the formation of apoptotic bodies or (D) based on the number of cells that lost the mitochondrial membrane potential. Values represent means of 3 experiments±S.D. *p<0.05.
and 7.5 μg/mL for 4 h showed significant enhancement of the caspase activity by 2.1 ± 0.3, 7.4 ± 0.6, and 9.8 ± 0.8 fold, respectively (p < 0.05), (Fig. 2A). TMX, at 10 μg/mL, induced the caspase activity by 7.9 ± 0.2 fold (p < 0.05).

KA Induced Nuclear Morphological Changes of MCF 7 Cells
Staining of KA-treated cells with a DNA binding dye, Hoechst 33258, showed distinct morphological characteristics including nuclei fragmentation and chromatin condensation (Fig. 3A). The chromatin structure was condensed progressively in a dose- and time-dependent manner, forming clusters against the nuclear periphery and/or crescent-like nuclei at higher concentrations. The apoptotic indexes, after 6, 9, and 12 h treatment with KA at 60 μg/mL, were 24.5 ± 3.6%, 32.4 ± 6.1% and 41.9 ± 2.7%, respectively. The apoptotic indexes at 80 μg/mL were 40.9 ± 8.3%, 46.7 ± 5.4% and 58.6 ± 2.7%, respectively (Fig. 3B).

KA Triggered DNA Fragmentation in MCF 7 Cells
Apoptotic cell death was further confirmed by studying the effect of KA on DNA fragmentation. Agarose gel electrophoresis of total genomic DNA showed that the treatment of MCF 7 cells with KA at 60 and 80 μg/mL caused an apparent dose-dependent ladder pattern, a characteristic feature of apoptosis (Fig. 2B).

KA Induced Loss of the Mitochondrial Membrane Potential in MCF 7 Cells
The effect of KA on the mitochondrial membrane potential of MCF 7 cells was investigated by staining the cells with a mitochondria probe, rhodamine 123. The treated cells appeared brighter than the untreated counterpart due to lower retention of the dye caused by the loss of the mitochondrial potential (Fig. 3C). The apoptotic indexes, after 6, 9, and 12 h treatment with KA at 60 μg/mL, were 20.4 ± 5.4%, 27.1 ± 4.5% and 31.7 ± 2.9%, respectively. At 80 μg/mL, the apoptotic indexes were 32.3 ± 6.8%, 43.8 ± 5.9% and 62.3 ± 9.7%, respectively (Fig. 3D).

KA Inhibited Migration and Invasion of MCF 7 Cells
The effect of KA on the migration of MCF 7 cells was examined by the wound healing assay. The percent of wound closure in the presence and absence of KA was calculated after 6, 9, 12, and 15 h, relative to the zero time. The results of the wound healing assay are as depicted in Fig. 4. The percentage of the wound closure after 15 h was almost 100% in the untreated cells, whereas the value in the treated cells was 69.7 ± 4.78%. The anti-invasive efficacy of KA was then assessed on matrigel. Figure 4C shows a representative photo of invasive cells in treated and untreated wells. At 15 μg/mL, KA significantly reduced the number of invasive cells through matrigel, by 52.1 ± 8.3%, (p < 0.05).

KA Obstructed MCF 7-Colony Formation
A 48 h treatment of MCF 7 cells with KA in the concentration range of 20–80 μg/mL caused dose dependent inhibition of the MCF 7 clonogenicity. The cell survival (%) graph for KA is depicted in Fig. 5. At concentrations of 60 and 80 μg/mL, KA inhibited the formation of MCF 7 colonies completely. At lower concentrations, KA significantly inhibited the formation of colonies; the SF at 40 and 20 μg/mL was 69.2 ± 6.4% and 28.1 ± 9.85%, respectively (p < 0.05). TMX, at 10 μg/mL, showed 100% inhibition.
Fig. 5. Clonogenic Cell Survival of MCF7 Treated with: (−) 1% Ethanol as a Negative Control, (+) 10µg/mL of TMX as a Positive Control, and Indicated Concentrations of KA

Data pooled from 3 experiments, with error bars representing the standard deviation of means. The plating efficiency (PE) was 37.3±8.4%. The results are presented as survival fraction (SF) %±S.D. (n=3). *p<0.05.

DISCUSSION

In this study we report the pro-apoptotic and antimetastatic properties of koetjapic acid (KA) towards the estrogen-dependent human breast cancer cell line (MCF 7). Triterpenes are a class of phytochemicals with strong anticancer activity toward a variety of cancers, including breast cancer.\(^{20,21}\) KA is a seco-A-ring oleane triterpene isolated from \textit{S. koetjape}.\(^{11,22}\)

In this study, KA exhibited a modest dose dependent anti-proliferative activity, with an IC\(_{50}\) value of 68.88±6.075 µg/mL. This result is consistent with previous reports which have studied the anti-proliferative capacity of KA.\(^{22,23}\) The apoptotic effects of KA were then investigated on early and late stages of the process. The results indicated that KA induced the executioner caspases. Activated caspases 3/7 are responsible for cleaving the cellular polypeptides, resulting in apoptosis-associated morphological changes, for example, the cleaving of β-catenin, E-cadherin and cytokeratin-18, causing disassembly of the cytoskeleton, loss of cell contact, disintegration, and fragmentation of cells.\(^{24,25}\) Moreover, caspase 3 cleaving of Lamin B and Acinus results in nuclear breakdown and chromatin condensation,\(^{24}\) all of which were observed after treatment with KA. Further evidence of apoptosis induction is the appearance of a clear DNA laddering pattern, which was observed upon treatment with KA. The extensive degradation of chromosomal DNA into nucleosomal units, as a DNA ladder, is a biochemical hallmark of apoptosis.\(^{26}\) Such fragmentation has been recognized with well-characterized apoptotic morphology in a huge number of cell types and under a variety of situations.\(^{27}\) After confirming the pro-apoptotic properties of KA, the next step was to explore whether

the mitochondria are involved in KA induced cell death. The mitochondrial pathway begins by activation of pro-apoptotic members of B-cell lymphoma cell 2 (Bcl-2), such as Bak and Bax proteins, which results in loss of the mitochondrial membrane potential. Subsequently, cytochrome c is released, which in turn activates caspase 3 and other caspases.\(^{28}\) In order to investigate the effect of KA on the mitochondrial membrane potential, we stained the cells with the mitochondria-specific lipophilic cationic dye, rhodamine 123. As a result of the positive charge, the dye accumulates selectively in cellular organelles with a high negative membrane potential, such as mitochondria.\(^{29}\) In the event of membrane potential loss, the dye will be repulsed and the cells will appear brighter. In this study, the MCF 7 treated with KA appeared brighter than the control cells in a time and dose dependent manner, which indicates that KA induced apoptosis by activating the mitochondrial pathway. Other terpenoids have been also reported to induce apoptosis by activation of the mitochondrial pathway.\(^{30–32}\)

In a previous study, we reported the capability of KA to inhibit estrogen receptor-negative MDA-MB-231 breast cancer and other cancer cell lines\(^{23}\); KA inhibited the proliferation of MDA-MB-231 and other cells with IC\(_{50}\) similar or lower than the values on MCF 7 cells. These results indicate that the cytotoxicity of KA against MCF 7 is not mediated by an anti-estrogenic pathway.

Tamoxifen is widely used as an adjunctive therapy for patients with breast cancer, to prevent breast cancer recurrence and metastasis. Its mechanisms of action, which include growth arrest and apoptosis induction, have been attributed mainly to its anti-estrogenic activity. However, many non-estrogenic receptor-mediated pathways have recently proved to be targets for tamoxifen, such as c-myc, tumor growth factor beta (TGF-β), mitogen-activated protein-kinases and others.\(^{33,34}\) The ability of KA to arrest MCF 7 growth and induce apoptosis could be attributed to these non-estrogenic mediated pathways, similarly to tamoxifen. Moreover, the induction of apoptosis of KA is mediated by a loss of mitochondrial membrane potential, which comes to an end of caspase 3 recruitment, the same mechanism of action was reported for tamoxifen.\(^{35}\)

Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver.\(^{35}\) Although less than 0.05% of circulating tumor cells have a potential to become stable metastasis,\(^{36}\) the vast majority of breast cancer-related deaths result from metastatic tumors. The current reports support the concept that breast cancer cells are inherently metastatic, and hence the disease should be considered systemic even at early stages.\(^{31}\) Accordingly, the discovery of metastasis inhibitors is coveted. Metastasis is a multi-step process that involves cell migration, invasion, colony formation and angiogenesis before a metastatic tumor can be formed. Herein, we examined the activity of KA against 3 major steps of this process. Previously, our research group reported the strong antiangiogenic activity of KA.\(^{12}\) Tumor cell migration is necessary at the initiation of the metastatic cascade, at which time the tumor cells leave the primary site and gain access to the body’s circulation, and also at the end of invasion, when cells enter the secondary site. KA significantly inhibited cell migration and invasion at the sub-cytotoxic concentration of 15µg/mL, which
indicated that the inhibitory effect is inherent rather than due to its anti-proliferative properties. After the migration and invasion stages, the metastasis process will be accomplished by cell attachment, survival, maybe dormancy, the formation of a colony and eventually further proliferation. KA also inhibited colony formation property even at a concentration less than the IC50. The significant reduction of number of colonies formed after 7d indicated that KA exhibited cytotoxic rather than cytostatic activity.

In conclusion, KA showed significant antimetastatic activities: it exhibited cytotoxic activity which is mediated via apoptotic mechanisms, caused a significant reduction in the clonogenic potential of MCF 7, and significant inhibition of MCF 7 migration and invasion. Taken together with its reported antiangiogenic properties, KA is a potential antimetastatic agent which may have use as an adjuvant therapy in breast cancer diseases.

Acknowledgements Zeyad D. Nassar would like to acknowledge USM for the USM Fellowship. This study was financially supported by a Universiti Sains Malaysia (USM) Research University Grant (Grant 1001/PFARMA/811144).

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