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Original article

Epoxy clerodane diterpene inhibits MCF-7 human breast cancer cell growth by regulating the expression of the functional apoptotic genes Cdkn2A, Rb1, mdm2 and p53

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ABSTRACT

Systematic analyses of plants that are used in traditional medicine may lead to the discovery of novel cytotoxic secondary metabolites. Diterpene possesses multiple bioactivities; here, epoxy clerodane diterpene (ECD) was isolated from *Tinospora cordifolia* (Willd.) stem and shown potential antiproliferative effect in MCF-7 human breast cancer cells. The antiproliferative effect of ECD on MCF-7 cells was systematically analyzed by cell and nuclear morphology, alterations in oxidative stress, and the expression of tumor suppressor and mitochondria-mediated apoptosis-related genes. We found that the IC_{50} value of ECD was 3.2 μM at 24 h and 2.4 μM at 48 h. We observed that the cytotoxicity of ECD was specific to MCF-7 cells, whereas ECD was nontoxic to normal Vero and V79 cells. ECD significantly triggered intracellular ROS generation even from the lower doses of 0.6 and 1.2 μM; and it is relative to higher dose of 2.4 μM. Further, we used 0.6 μM, 1.2 μM and 2.4 μM as experimental doses to analyze the relative dose-dependent effects. Nuclear staining revealed that cells treated with the 2.4 μM dose exhibited characteristic apoptotic morphological changes and that 46% of the cells were apoptotic and 4% were necrotic after 48 h. ECD significantly increased the expression of mitochondria-dependent apoptotic pathway-related genes after 48 h; we observed significantly ($p \leq 0.05$) increased expression of CYP1A, GPX, GSK3β and TNF-α and downregulated expression of NF-κB. ECD also increased the expression of tumor suppressor genes such as Cdkn2A, Rb1 and p53. In addition, we observed that ECD treatment significantly ($p \leq 0.001$) upregulated the expression of apoptotic genes such as Bax, cas-3, cas-8, cas-9 and p21 and downregulated the expression of BCL-2, mdm2 and PCNA. In conclusion, ECD regulates the expression of Cdkn2A, p53 and mdm2 and induces apoptosis via the mitochondrial pathway in MCF-7 human breast cancer cells.

1. Introduction

Breast cancer is a major health problem in women. It is the main cause of death for women between the ages of 40 and 50 years [1]. Breast cancer is responsible for 23% of the total cancer diagnoses and 14% of deaths in women worldwide [2]. The main difficulty in preventing cancer progression is the increased resistance, low specificity and high toxicity of commercially available drugs [3]. Cancer cells must circumvent antiproliferative signals that negatively regulate growth and proliferation. In cancer cells, stress response against hyper-proliferation tends to be suppressed, leads to continued proliferation of cells which stimulate overactive mitogenic signals [4]. However, upon hyper-proliferation signals in the non-cancerous cells leads to increased expression of tumor suppressor genes $p16^{Ink4a}$ (pRb1) and $p14^{ARF}$ (Cdkn2Al; cyclin-dependent kinase inhibitor 2A), which govern the antiproliferative functions [5]. $p14^{ARF}$ neutralizes the ability of mdm2 to promote p53 degradation, leading to more p53 accumulation and functional stabilization [4]. Zhao et al. [6] have reported that the suppression of $p14^{ARF}$ transcription prevented the deactivation of mdm2 by $p14^{ARF}$, blocking p53 from continuing cellular growth.

p53 is a key regulator of cell cycle checkpoints and apoptosis, controlling the transcription of genes related to hyper-proliferation, including p21 and BAX [7]. p21 is an important cellular checkpoint molecule for the inhibition of the activities of a range of cyclin-DKs [8]. The $p14^{ARF}$/mdm2/p53 pathway is therefore critical

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for normal cell cycle progression [9]. Thus, the tumor suppressor proteins of the *INK4a/Arf* locus function in distinct anticancer pathways, as $p16^{INK4a}$ directly regulates Rb1, while $p14^{Arf}$ directly regulates p53 and indirectly regulates pRb. Cancer cells can avoid this control step by losing the physiological function of pRb, which controls all antiproliferative signals [10]. Frequent mutations in $p14^{ARF}$, mdm2 and p53 or the loss of their expression have been identified in tumors and malignant mesothelioma [11,12]. Accordingly, the inhibition of cell growth and invasiveness by stabilizing p53 via retinoblastoma (Rb)/$p14^{ARF}$/mdm2 signaling is one potential method of tumor suppression therapy [13].

The success of cancer chemotherapy depends on the development of drugs that selectively destroy tumor cells or at least limit their proliferation without causing severe side effects [14]. Numerous anticancer drugs have been developed from plants, such as vinblastine and vincristine from *Catharanthus roseus*, rohitukine from *Dysoxylum binectariferum,* and camptothecin derived from *Camptotheca acuminata*, and there is no reason to think that the list is complete [15]. *Tinospora cordifolia* (Willd.) Miers (Guduchi) is a well-known medicinal plant, and a series of its chemical constituents have been identified, of which diterpenoids and alkaloids in particular displayed a broad spectrum of biological activities, such as anti-inflammatory, anti-nociceptive, and anticancer activities [16]. We recently extracted and identified a clerodane-derived diterpenoid, (5R,10R)-4R,8R-dihydroxy-2S, 3R:15, 16-diepoxycleroda-13 (16), 17, 12S:18,1S-dilactone [epoxy clerodane diterpene-(ECD)] from *T.cordifolia* that showed antiproliferative activity against hepatocellular carcinoma [17]. Previously, esculentin A and esculentin B, isolated from *Casearia esculenta* and belonging to the clerodane diterpene group, demonstrated anticancer activity [18]. ECD derivatives isolated from *Croton lechleri* showed anti-tumor and apoptotic activities in prostate cancer [19]. In the present study, the cytotoxic and tumor suppressive potential of ECD via the regulation of the cdkn2A/mdm2/p53 pathway in MCF-7 breast cancer cells was analyzed. In addition, we aimed to explore the systematic action of ECD on tumor suppression and cancer cell senescence in MCF-7 cells.

2. Materials and method

Propidium iodide (PI), acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Roswell Park Memorial Institute medium (RPMI-1640) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The DeadEnd TUNEL Assay Kit was procured from Promega (Madison, WI, USA). The QuantiTect Primer Assay, Fast Lane Cell cDNA Kit and QuantiFast SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). All other chemicals used were molecular biology research grade.

2.1. Extraction, isolation and identification of ECD from T. cordifolia

The stems of T. cordifolia plants were shade dried, powdered (1 kg) and extracted with alcohol by a cold percolation method. The extract was evaporated to dryness in a vacuum, and 25 g of the extract was chromatographed using silica gel (100–200 mesh). Compounds were eluted from the column by increasing the solvent polarity (hexane to methanol). When the solvent polarity was increased to chloroform: methanol ratio of 19:1, a white-colored compound was eluted and confirmed as a terpenoid [20]. Upon repeated crystallization from methanol, the compound yielded transparent crystals. A crystal of dimensions $0.22 \times 0.20 \times 0.16$ mm was selected for data collection (Indian Institute of Technology, Madras) using a graphite radiation monochromator (Bruker, 1999).

2.2. Cell culture

The noncancerous Vero cell line and the V79 hamster lung fibroblast cell line were obtained from the National Center for Cell Sciences, Pune, India. The MCF-7 human breast cancer cell line was obtained from Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animals in Life Science Education, Bharathidasan University, Tiruchirappalli-620024, India, as a gift. The MCF-7 cells were cultured as a monolayer in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 100 μg/ml of the antibiotic streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 (Thermo Scientific, USA).

2.3. In vitro cytotoxicity assay using MTT

Cell viability was assessed based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase enzymes of viable cells to a purple formazan product [21]. Briefly, noncancerous Vero cells, V79 Chinese hamster lung fibroblasts and MCF-7 cells were plated at a density of 1×10^4 cells/mL in 96-well plates. After overnight growth, the cells were treated with increasing concentrations of ECD (0, 0.125, 0.25, 0.5, 0.625, 1.25, 2.5, 5, 7.5, 10 and 20 μM) and maintained for 24 h and 48 h. The compound was crystalline, white colored and soluble in a solution of 10% (v/v) DMSO in PBS, and no color interference was observed. The final concentration of the solvent was always <0.2%. For the positive control, the same concentrations of doxorubicin and tamoxifen were used. After incubation, the medium was replaced with 100 μL of fresh medium, and 10 μL of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) was immediately added to each well. The plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The plates were centrifuged, and the purple formazan product was dissolved by the addition of 100 μL of DMSO to each well. The absorbance was measured at 570 (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, CA, USA). Quadruplicate samples were run for each concentration of ECD in three independent experiments. The viability of the Vero, V79 and MCF-7 cells was expressed as the percent viability of treated cells compared with untreated cells. The percentage of toxicity was calculated from the data using this formula: [(Mean OD of untreated cells – Mean OD of treated cells)/(Mean OD of untreated cells)] × 100.

2.4. Measurement of intracellular ROS

The production of intracellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [22]. DCFH-DA passively enters the cell, where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted 500-fold in HBSS without serum or other additives to yield a 20 μM working solution. After 24 h of exposure to ECD (0.6 μM, 1.2 μM and 2.4 μM), MCF-7 cells in a 24-well plate were washed twice with HBSS and then incubated in 2 mL of the DCFH-DA working solution at 37 °C for 30 min. In addition, MCF-7 cells were treated with either ECD and 20 mM *N*-acetyl cysteine or 20 mM *N*-acetyl cysteine alone (positive control). The fluorescence was then determined at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a microplate reader.