Free Radical Biology & Medicine 52 (2012) 1423–1436

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Deoxyelephantopin impedes mammary adenocarcinoma cell motility by inhibiting calpain-mediated adhesion dynamics and inducing reactive oxygen species and aggresome formation

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ARTICLE INFO

Article history:
Received 18 June 2011
Revised 13 January 2012
Accepted 21 January 2012
Available online 2 February 2012

Keywords:
Deoxyelephantopin
Sesquiterpene lactone
Paclitaxel
Mammary adenocarcinoma
Reactive oxygen species
Calpain
Focal adhesion
Cancer cell motility
Free radicals

ABSTRACT

We previously showed that deoxyelephantopin (DET), a plant sesquiterpene lactone, exhibits more profound suppression than paclitaxel (PTX) of lung metastasis of mammary adenocarcinoma TS/A cells in mice. Proteomics studies suggest that DET affects actin cytoskeletal protein networks and downregulates calpain-mediated proteolysis of several actin-associated proteins, whereas PTX mainly interferes with microtubule proteins. Here, DET was observed to significantly deregulate adhesion formation in TS/A cells, probably through inhibition of m-calpain activity. Epithelial growth factor (EGF)-mediated activation of Rho GTPase Rac1 and formation of lamellipodia in TS/A cells were remarkably suppressed by DET treatment. Further, DET impaired vesicular trafficking of EGF and induced protein carbonylation and formation of centrosomal aggregates in TS/A cells. DET-induced reactive oxygen species were observed to be the upstream stimulus for the formation of centrosomal ubiquitinated protein aggregates that might subsequently restrict cancer cell motility. PTX, however, caused dramatic morphological changes, interfered with microtubule networking, and moderately inhibited calpain-mediated cytoskeletal and focal adhesion protein cleavage in TS/A cells. This study provides novel mechanistic insights into the pharmacological action of DET against metastatic mammary cell migration and suggests that modulation of oxidative stress might be a potential strategy for treatment of metastatic breast cancer.

Because of better and earlier diagnosis and postoperative adjuvant therapies, the survival rate among breast cancer patients has improved greatly in recent years [1]. However, metastatic breast tumor remains the leading cause of death among patients diagnosed with the recurrent disease. Taxanes are currently considered to be the most active therapeutic agents in the management of metastatic breast cancer [2]. Paclitaxel (PTX), a diterpene alkaloid initially isolated from *Taxus brevifolia,* was the first taxane to show activity in breast cancer. It acts by targeting β-tubulin to stabilize microtubule dynamics, subsequently inducing apoptosis of cancer cells [2,3]. Nevertheless, emerging clinical resistance to taxanes after their extensive use in chemotherapy worldwide has reduced their effectiveness in the treatment of breast cancer [4]. Recently, accumulating evidence has shown that various phytocompounds possess chemopreventive or chemotherapeutic properties, suggesting their potential for use in combination with chemotherapy as effective treatments for breast cancer [5]. One such potent antitumor phytocompound is the major germacranolide sesquiterpene lactone deoxyelephantopin (DET), from the traditional medicinal herb *Elephantopus scaber* L. [6,7]. When we investigated it in parallel with PTX, we observed that DET profoundly suppressed mammary carcinoma metastasis in vitro and in vivo [8]. Further, investigation using two-dimensional differential in-gel electrophoresis indicated that DET and PTX act differently on the cytoskeleton: DET exerted its effect mainly on the actin-based cytoskeletal system, whereas proteins upregulated by PTX were associated mainly with regulation of microtubules in TS/A cells, consistent with the well-known function of PTX as a microtubule-stabilizing agent in mammary cancers [9].

Intriguingly, cleaved forms of several focal adhesion- and membrane attachment-associated cytoskeletal proteins—focal adhesion

Abbreviations: DET, deoxyelephantopin; PTX, paclitaxel; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; ROS, reactive oxygen species; FAK, focal adhesion kinase; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; MG132, carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; TG, thapsigargin; NAC, *N*-acetyl-L-cysteine; LPA, oleoyl-L-α-lysophosphatidic acid; DHE, dihydroethidium; PDI, protein disulfide isomerase; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; RFP, red fluorescent protein; GST, glutathione *S*-transferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ESI–MS/MS, electrospray ionization–tandem mass spectrometry; t-BOC-LM-CMAC, 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionine amide; PIP_2, phosphatidylinositol 4,5-bisphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; UPS, ubiquitin proteasome system; MTOC, microtubule-organizing center.

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doi:10.1016/j.freeradbiomed.2012.01.020

kinase (FAK), spectrin α2, filamin A, and ezrin—were specifically downregulated in DET-treated cells [9]. These proteins are known to be substrates of calpains. Calpains are calcium-dependent cysteine proteinases that have been widely studied for their roles in several key aspects of cell motility. The most well characterized calpains are the ubiquitously expressed μ-calpain and m-calpain [10]. In general, calpains cleave substrate proteins localizing near the cell membrane and cytoskeleton in a limiting manner and thus function as biomodulators of cell physiology [11]. M-calpain, but not μ-calpain, is required for proteolysis of the cytoskeletal and focal adhesion proteins, such as FAK and spectrin [12], that were responsive to DET treatment in TS/A cells. Among the many proteases involved in cell motility, the calpains play an essential role in regulating migration and invasion, both of which are involved in the development of metastases. A significant level of m-calpain was observed in metastatic prostate cancer [13], and the upregulation found in breast cancer was correlated with increased invasive properties of tumors [14]. Recently, calpains have been reported to be markers of tumor aggressiveness and potential targets for limiting development of rhabdomyosarcoma tumor [15].

Rho-family GTPases are the key regulators of organization of the actin cytoskeleton. The commonly known members of this family, Rac1, Cdc42, and RhoA, regulate the formation of lamellipodia, filopodia, and stress fibers, respectively, in response to extracellular signals [16]. In an attempt to account for the specificity of the DET-mediated inhibitory effect on actin-based cell migration in this study, we examined two calpain-associated cytoskeletal structures, membrane protrusion and focal adhesion plaques, in parallel compared with the action of PTX. Failure of the ubiquitin–proteasome system causes protein aggregates termed “aggresomes” in the pericentrosomal area in cells [17]. In addition, endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) induction were also reported to trigger apoptosis of cancer cells treated with bortezmib (also known as PS-341), the first proteasome inhibitor approved for treating relapsed multiple myeloma [18]. We observed that ROS induced in DET-treated cells can regulate aggresome formation. In contrast to the massive disruption of microtubules induced by PTX, DET caused cancer-cell-specific inhibition of the actin cytoskeleton possibly through deregulation of calpain activity and inhibition of Rac1-mediated lamellipodia formation. Taken together with evidence of the ROS-mediated inhibitory effect of DET treatment on mammary tumor cell migration, these findings suggest that modulation of oxidative stress might be a potential strategy for the treatment of metastatic breast cancer.

Materials and methods

Cell lines and cell cultures

TS/A cells, a murine mammary adenocarcinoma cell line, and H184B5F5/M10, a noncancerous human mammary epithelial cell line (ATCC), were grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), in a humidified 5% CO_2 incubator at 37 °C.

Isolation and structure elucidation of DET

The protocols for extraction and identification of DET from *E. scaber* L. (Asteraceae) (voucher specimen ES001 deposited in the Agricultural Biotechnology Research Center, Academia Sinica, Taiwan) followed the method of Huang *et al.* [8]. The structure of DET was elucidated by electrospray ionization mass spectrometry (ThermoFinnigan LCQ) and ^{1}H and ^{13}C NMR (Brüker Advance 500 AV) spectrometry and confirmed by comparison of the spectral data with previously published results [19]. The molecular weight of DET is 344. The compound purity was >98% as judged by LC/MS and NMR spectral data.

Chemicals and reagents

Dimethyl sulfoxide (DMSO), PTX, carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132), thapsigargin (TG), ionomycin, fibronectin, *N*-acetyl-L-cysteine (NAC), Y27632 (RhoA inhibitor), oleoyl-L-α-lysophosphatidic acid (LPA), and epidermal growth factor (EGF) were purchased from Sigma–Aldrich. Fluorogenic calpain substrate II, calpeptin, purified recombinant rat m-calpain, and Rac1 inhibitor were purchased from Calbiochem. t-BOC-Leu-Met and dihydroethidium (DHE) were purchased from Invitrogen. All other chemicals and solvents were of reagent or high-performance liquid chromatography grade. Primary antibodies against actin (Chemicon, Millipore), β-tubulin (Epitomics), FAK, Rac1, Cdc42 (BD Biosciences), vinculin, γ-tubulin (Sigma–Aldrich), ezrin, and protein disulfide isomerase (PDI) (Cell Signaling Technology) were used. All other antibodies were from Santa Cruz Biotechnology.

Time-lapse microscopy

A 60-mm-diameter culture plate was coated with 10 μg/ml fibronectin for 1 h and then seeded with TS/A cells in DMEM containing 10% serum. Twelve hours after seeding, time-lapse microscopy experiments were performed on an inverted Zeiss Axiovert 200 M microscope equipped with an environmental chamber with phase-contrast optics (images taken every 30 min). Using the object-tracking application of Metamorph software (Molecular Devices), an average of 12 subsequent cell centroid displacements/30 min between two consecutive images were evaluated as cell velocities of migration. In total, cell trajectories were recorded for 18 h. These assays were made in treatments with vehicle (DMSO, 0.05%), DET (5.0 μM), and PTX (2.0 μM) added at the beginning of the time-lapse.

Immunofluorescence cell staining

TS/A cells were seeded on 12-mm glass slips in 24-well plates coated with fibronectin/poly-D-lysine for 16 h and then treated with vehicle (DMSO, 0.05%), DET (5.0 μM), PTX (2.0 μM), or calpeptin (20 μM) for 6 h. Cells were fixed with 4% paraformaldehyde and then permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100. For immunostaining of γ-tubulin, cells were fixed in −20 °C methanol for 5 min. After being rinsed twice with PBS, the cells were blocked with PBS containing 3% bovine serum albumin and then stained with primary antibody and secondary antibody (Cy3/FITC conjugated; Jackson ImmunoResearch Laboratories) at a dilution of 1:200. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). Fluorescence imaging was performed and captured on a Zeiss LSM510 META laser scanning confocal microscope equipped with 100×/1.4 oil plan-Apochromat in multitrack channel mode. Image processing was performed using a confocal laser scanning microscope (LSM 510 version 4.2; Zeiss).

Fluorescence imaging

For F-actin staining, cells were fixed with 4% paraformaldehyde after treatments and then permeabilized with PBS containing 0.2% Triton X-100. After being rinsed with PBS, fixed cells were incubated in buffer with rhodamine–phalloidin at room temperature for 30 min and washed again with PBS. For imaging of cellular organelles, Golgi apparatus and early endosomes were visualized in cells transiently transfected with GFP-tagged GalNAc-T2 and RFP-tagged Rab5a using Cell-light Reagents BacMam 2.0 (Invitrogen) according to the manufacturer's instructions. For EGF internalization, cells were serum-starved overnight and then incubated at 37 °C in DMEM with 1% serum and 2 μg/ml rhodamine EGF (Invitrogen) for 15 min. After being labeled, the cells were washed twice with PBS and fixed. The nuclei were stained with DAPI. The cellular staining on actin filaments in TS/A cells was viewed and captured on a Nikon Eclipse E800