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Plumbagin triggers DNA damage response, telomere dysfunction and genome instability of human breast cancer cells

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ABSTRACT

Aim: Natural plant products are increasingly being used in cancer therapeutic studies due to their reduced normal cell toxicity. In this study, the anti-cancer properties of plumbagin, a naphthoquinone derivative extracted from the roots of Plumbago, were evaluated in breast cancer cells.
Methods: To evaluate the effects of plumbagin on breast cancer cell types, we employed a variety of techniques comprising cell viability, cell cycle assay, comet assay, western blotting, immunocytochemistry, measurement of telomerase activity, telomere restriction fragment length, quantitative fluorescence in situ hybridisation, along with gene expression analysis of untreated cells.
Results: Plumbagin treatment induced cytotoxicity in human breast cancer cells along with cell cycle arrest, DNA damage and cell death leading to apoptosis. Plumbagin was also found to suppress the telomerase activity in cancer cells accompanied by telomere attrition. Telomere shortening was corroborated by reduced telomere fluorescence on chromosome ends and genome instability.
Conclusion: Together, these findings may suggest the application of plumbagin as adjuvant modality in breast cancer therapeutics.

1. Introduction

Breast cancer, as the foremost cause of cancer death among women, is a heterogeneous disease with a variety of genetic and molecular alterations driving its growth, survival and response to therapy [1]. Based on the gene expression profile patterns of hormone receptors oestrogen(ER), progesterone (PR) and human epidermal growth factor receptor (HER2), there are three imperative subtypes of breast cancer: luminal, HER2$^+$ positive and basal like breast tumours [2]. Luminal tumours, which express ER and PR receptors and HER2$^+$ tumours, could be controlled through their respective targets. A serious challenge is met for the highly aggressive basal-like tumours, also known as triple-negative breast cancers (TNBC), lacking expression of all three receptors. With regard to this deficiency, no molecular-based targeted treatment is available for TNBC at present besides chemotherapy as the only accessible systemic therapy, highlighting the fact that developing enhanced therapy for TNBC is one of the most crucial concerns of modern breast cancer research modalities [2,3]. However, current approaches to TNBC targeted therapy are mainly focusing on the development of new investigational drugs, which directly target DNA repair pathways through DNA-binding or DNA-damage potentiation [4]. Telomerase is a hallmark of cancer and majority of the human cancers including breast cancer express this enzyme. Therefore, inhibition of telomerase would be an attractive target for breast cancer treatment [5,6]. Telomerase enzyme activity has been found in majority of cancer cells but not in adjacent normal cells to mediate the telomere length maintenance mechanism during cancer cell proliferation; and accordingly leading to unlimited replicative potential [7].

Natural plant chemicals called phytochemicals are known to be promising anti-cancer agents comprising the advantage of being less toxic to normal cells as compared to most of traditional chemotherapeutic drugs [8]. Phenolic plant compounds are identified to have both preventive and therapeutic potential in combating cancer. It is interesting that their anti-cancer effects resemble consequences of certain fatty acids such as omega-3

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polyunsaturated fatty acids and conjugated linoleic acids [9], which were reported to be potential protective factors in preventing breast cancer [10]. Amongst phenolic agents, 1,4-naphthoquinones are characterised with potent anti-cancer abilities [11,12].

Plumbagin is a 1,4-naphthoquinone derivative and like other phenolic compound members of this group is known to possess anti-tumour properties [12–16]. This compound was originally extracted from the roots of Plumbago species, which have been credited with notable medicinal properties, worldwide [17,18]. Plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone, is a naturally occurring structural analogue of vitamin K3 (menadione; 2-methyl-1,4-naphthoquinone), which, both were reported to be substrates and inhibitors of the breast cancer resistance protein, also known as multidrug resistance-linked ATP binding cassette drug transporter, ABCG2 [19]. Both plumbagin and vitamin K3 have manifested to be potential anti-cancer agents against breast cancer via the mitochondria-related pathway [20,21]. Plumbagin was revealed to induce cell cycle arrest at G2/M phase and autophagy by restraining the AKT/mammalian target of rapamycin and to inactivate NF-κB and Bcl-2, leading to apoptosis in breast cancer cells [22,23].

Previous studies on plumbagin have mainly focused on molecular signalling pathways and there is still no information about the role of plumbagin in telomere/telomerase dynamics and genome instability of breast cancer cells. We had earlier established that plumbagin induces cytotoxic and genotoxic effects in human brain tumour cells and for the first time studied its contribution to telomere attrition and inhibition of telomerase activity [24]. In this study, we investigated a variety of anti-cancer effects of plumbagin treatment in the breast cancer cell types MDA-MB-231 (TNBC) and MCF-7 (ER+) as compared to the relatively normal MCF-10A cells. In this study, we aimed to further investigate the inhibitory effects of this phytochemical on telomerase activity and telomere length, along with its consequences on DNA strand damage and genome instability in breast cancer cells.

2. Materials and methods

2.1. Cells, cell culture conditions and drug treatment

Breast cancer cell types MDA-MB-231 (TNBC) and MCF-7 (luminal tumour)cells were grown in RPMI 1640 (Gibco, Invitrogen Corporation), supplemented with 10% foetal bovine serum (FBS) and 0.5% penicillin/streptomycin. Human immortalised normal breast epithelial cells (MCF-10A) were grown in complete mammary epithelial growth medium (MEGM) (Clonetics, Lonza, USA) supplemented with bovine pituitary extract (BPE) (Lonza, USA), 10% FBS and 0.5% penicillin/streptomycin. All cells were grown and incubated at 37 °C in a humidified 5% CO_2 incubator. Plumbagin (Sigma-Aldrich, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) at the final concentration of 0.1% [v/v] to prepare 100 mmol/L stock solution from which working concentrations were prepared later. Growing cells were treated with a range of 0–4 μmol/L of plumbagin for 48 h following which the inhibitory concentration at 50% cell viability (IC_{50}) was obtained and selected for further studies. Throughout the study, treatment conditions were 48 h with IC_{50} doses and32 d with two third of IC_{50} doses, for short term and the long-term studies, respectively. The drug vehicle DMSO is considered as zero doses in the findings.

2.2. Cell viability

Through three independent sets of experiments, cells were seeded in 6-well culture plates and following treatment with plumbagin, they were washed with 1X phosphate buffered saline (PBS) and incubated for 15 min at 37 °C with the crystal violet solution (0.75% crystal violet in 50% ethanol:distilled water, 1.75% formaldehyde and 0.25% NaCl), which stains the DNA by binding electrostatically to nuclear proteins. Thereafter, the non-adherent cells were washed away by PBS. The dilution of 1% sodium dodecyl sulphate (SDS) in PBS was added to lyse the cells and solubilise the dye. Difference in cell density was displayed on an ELISA reader by the quantity of crystal violet taken at 595 nm absorbance. For morphological observations, normal light microscope was utilised. After 48 h of treatment with plumbagin, images of cell morphology were captured at and 200× magnifications.

2.3. Clonogenic assay

To determine the effects of plumbagin on the colony formation ability, through four independent sets, 2×10^3 cells were seeded and were allowed to grow for 14 d in fresh medium after 48 h treatment. Colonies were then stained with crystal violet solution (Sigma, USA) (as described above), and dried at room temperature. Thereafter large colonies (>50,000 cells) were detected and counted as survived number of colonies.

2.4. Migration assay

Cell migration ability after plumbagin treatment was evaluated by migration assay, also known as wound healing assay. According to previous-described protocol [25]. Briefly, 60-mm dishes were marked on the outer bottom as reference points and cells were dispersed gently and seeded equally among the plate. After 6 h of adherence, the confluent monolayer of cells was scrapped manually in straight lines according to the reference point and a wound was generated by a p200 pipette tip. In 3 independent sets, cells were treated with plumbagin and images were acquired at time 0 and 48 h of incubation with the drug. The photographed regions were analysed quantitatively by the Image J Molecular Imaging Software [26] and the migration of cells after 48 h was identified by the number of cells that crossed into the wound area from their reference point at time zero.

2.5. Cell cycle analysis

Following treatment with plumbagin, cells were collected, fixed in 70% ethanol: 1X PBS, resuspended in 0.4 mL of 1xPBS containing propidium iodide (PI) (Sigma, USA), RNase A (Roche, USA) and 0.1% Triton X (Biorad, USA) (2 mg propidium iodide and 2 mg RNaseA/100 mL 1X PBS), incubated in the dark for 30 min at 37 °C. For each treatment, two independent sets were performed and for each 10,000 events per sample were captured and analysed using flow cytometry (FACSCalibur™, Becton Dickinson, USA) at 488 nm excitation λ and 610 nm emission λ. Obtained data was analysed using Summit 4.3 software (Beckman Coulter, USA).

2.6. Western blot analysis

For protein estimation, total cellular proteins, following plumbagin treatment, were isolated using lysis buffer (distilled water containing 50 mmol/L Tris HCL, pH 8.5 mmol/L EDTA, 0.15 mol/L sodium chloride, 0.5% NP40, 0.5 mmol/L DTT, 1 × PhosSTOP (Phosphatase inhibitor cocktail tablets, Roche, Germany), 1 × Protease cocktail tablet (Roche, Germany)), following which lysates were rotated at 4 °C at 14,000 rpm for 30 min. Protein concentration was determined by the colorimetric Bradford assay (Bio-Rad,USA) with bovine serum albumin (BSA) as a standard. Equal amounts of protein were resolved by SDS–PAGE at different gel densities (6–12%) and transferred to polyvinylidene difluoride