Original Article

Plumbagin shows anticancer activity in human osteosarcoma (MG-63) cells via the inhibition of S-Phase checkpoints and down-regulation of c-myc

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Abstract: Objective: Plumbagin, a naphthoquinone constituent of Plumbago zeylanica L. (Plumbaginaceae), has been extensively studied for its pharmacological activities and reported to show a good anti-cancer activity in different human cancer cell lines. It is known to exhibit proapoptotic, antiangiogenic and antimetastatic effects in cancer cells. Plumbagin is also known to inhibit NF-κB, JNK (Hsu), PKCε, and STAT-3. However, the anti-proliferative activity and their core molecular mechanisms have been poorly determined. Methods: Human osteosarcoma (MG-63) cells were exposed to plumbagin and the anti-proliferative activity was evaluated by MTT assay. The mechanism of action for the growth inhibitory activity of plumbagin on MG-63 cells was evaluated using flow cytometry for cell cycle distribution, and western blot for assessment of accumulation and phosphorylation of potential target proteins. Furthermore, morphology of MG-63 cells was assessed after treatment with Plumbagin. Results: Plumbagin has significantly induced growth inhibition against osteosarcoma MG-63 cells, primarily by S-phase cell cycle arrest which is confirmed by the down regulation of cyclin A and CDK2 protein levels determined by western blot analysis. It was also found that plumbagin has triggered the DNA damage in MG-63 cells, subsequently initiating the arrest in S-phase, which is evident by the up-regulation of phosphorylated p53 and histone. Furthermore, plumbagin resulted in the down-regulation of c-myc protein expression in the MG-63 cells. Conclusion: Plumbagin has triggered DNA damage and had induced S-phase arrest in MG-63 cells, suggesting it to be a potential compound in treatment against malignant human osteosarcoma.

Keywords: Osteosarcoma, plumbagin, phosphorylation, cyclin A, CDK2, p53

Introduction

Cancer is revolving out as a very dreadful disease in modern days and combating cancer is a major public health issue as, the resistance to apoptosis by tumor cells displays obstacle in treating all malignancies, hence, novel targeted therapies are much in demand. Osteosarcoma is been documented for almost centuries and it is the most common primary, non-haemopoietic malignant tumour of the skeletal system accounting for almost 20% of all primary malignant bone tumours [1]. Though osteosarcoma is very rare among young children (0.5 cases per million per year in children <5 y), the age distribution for osteosarcoma is found as bimodal and is propensity to develop in adolescents and young adults [2-4] where 60% of tumour is found in patients younger than 25 years of age and only 13% to 30% are in patients who are older than 40 years [5]. Osteosarcoma can transpire in any bone, but mostly tumours are found to originate in the long bones of the appendicular skeleton, near metaphyseal growth plates, especially the distal femur, followed by the proximal tibia and proximal humerus [6]. The skull and jaw and pelvis are reported to be other significant locations for tumours [7]. Various signaling pathways such as, Akt/PI3K/MAPK signaling are been reported to be involved in elevation of many tumors and also in cell survival. Sequential activation of cyclin-dependent kinases is involved in cell cycle regulation in eukaryotic cells and deregulation of these protein expressions is commonly detect-
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Figure 1. Chemical structure of plumbagin (Molecular formula: 5-hydroxy-2-methyl-1,4-naphthoquinone, Molecular weight: 188.18.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), an yellow pigment is natural occurring quinonoid constituent (Figure 1). This is primarily found in the plants of Ancestrocladaceae, Dioncophyllaceae and Plumbaginaceae families and is isolated from Plumbago zeylanica root [18]. However widely used in traditional medicine, plumbagin exhibits various biological activities such as anti-atherogenic, anticancer, anti-proliferative, cardiotoxic, chemopreventing, hepatoprotective and neuroprotective effects. It also exhibits pro-apoptotic and radiosensitizing activities in different tumor cells and animal models both in vitro and in vivo [19-23]. Sandur et al. [23] has reported that plumbagin is an efficient inhibitor of NF-κB activation, where plumbagin suppressed NF-κB in various cancer cells, ultimately leading to the suppression of downstream NF-κB-regulated gene products and also other gene related activities. Considering the various activities of plumbagin, we investigated the anticancer effect of plumbagin on human osteosarcoma (MG-63) cell lines and hence its effect on cell proliferation and c-myc signalling in these cells.

Materials and methods

Chemicals and reagents

Anti-β-actin antibody, bovine serum albumin (BSA), propidium iodide (PI), ribonuclease A (RNase A), trichloroacetic acid (TCA), sulforhodamine B (SRB) and plumbagin were obtained from Sigma-Aldrich, St.Louis, MO, USA. Fetal bovine serum (FBS), RPMI 1640, trypsin-EDTA and antibiotic solution were purchased from GIBCO-BRL (Grand Island, NY, USA). Antibodies against Cyclin A, Cdk2, c-myc and CDK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-H2A.X Ser (139) and P-p53 (Ser15) were obtained from Cell Signaling (Danvers, MA, USA).

Cell culture

MG-63 human osteosarcoma cells were obtained from American Type Culture Collection (Manassas, VA) and cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS) (10%) and antibiotics (PSF; 100 units/mL penicillin G sodium, 100 μg/mL streptomycin and 250 ng/mL amphotericin B). The MG-63 human osteosarcoma cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere.

Cell proliferation assay

Effect of plumbagin on the proliferation of MG-63 cells was assessed using 3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [24]. Briefly, the MG-63 cells were seeded onto 96-well plates where 5x10^4 cells/well cell density was maintained. The cells were then subjected to different concentrations of plumbagin or vehicle control (DMSO) for about 72 h. After treatment, cell growth was analyzed by addition...
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Figure 2. Cytotoxic effect of plumbagin on MG-63 human osteosarcoma cells lines. Data is represented as mean ± SD of three different experiments, *P<0.05 and **P<0.01 (Students t-test).

of MTT (100 μL of 1 mg/mL MTT). Following 4 h of incubation, DMSO was added to lyse the cells and dissolve the formed purple formazan crystals. The formazan product’s absorbance was determined at λ max of 595 nm using a Tecan Spectra Flou spectrophotometer (MTX Lab Systems Inc., Vienna, VA). By plotting the percentage survival of cells against the concentration of plumbagin, the IC<sub>50</sub> values were obtained from the sigmoidal curve.

Cell cycle analysis

MG-63 human osteosarcoma cells at a density of 5×10<sup>3</sup> cells per 100 mm were plated in culture dishes and were incubated for 24 h. Fresh growth media containing different concentrations of plumbagin was added to culture dishes. The cells were harvested following 24 h incubation and were fixed with 70% ethanol overnight at 4°C. The fixed cells were washed with PBS and incubated with RNase A (50 μg/mL) and propidium iodide (50 μg/mL) containing staining solution for 30 min at room temperature. Cell cycle distribution was analyzed using Becton Dickinson (San Jose, CA) flow cytometer and at least 10,000 cells were analyzed for each experimental condition. Data analysis was performed using Cell Quest cell cycle analysis software.

Western blot analysis

MG-63 human osteosarcoma cells were exposed to different concentrations of plumbagin for 24 h and after incubation, cells were lyled and protein concentration was determined by Lowry’s method. The proteins (40-45 μg) were then subjected to SDS-PAGE. Briefly, the obtained proteins were transferred onto PVDF membranes by electro-blotting and membranes were treated for 1 h with blocking buffer (5% non-fat dry milk) and were incubated with antibodies overnight at 4°C. Membranes were washed thrice for 5 min with PBST and bands were visualized by HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) using LAS-3000 Imager (Fuji Film Corp., Japan). Antibodies were diluted as recommended by the manufacturers for western blotting.

Statistical analysis

All the experiments were performed in triplicates and the obtained values expressed as mean ± standard deviation (SD) for the indicated number of independently performed experiments and were analyzed using Student’s t-test. Values of P<0.05 were considered statistically significant.

Results and discussion

The identification and development of novel chemotherapeutic agents that can increase survival rates and lower the toxic side effects is vital. Recent researches have demonstrated the potent anti-neoplastic activity of plumbagin on lung and breast cancer cells [25, 26]. In this study we examined the antineoplastic activity of plumbagin against human osteosarcoma cell line MG-63.

Effect of plumbagin on MG-63 cell proliferation

The anti-proliferative effect of plumbagin (Figure 2) against MG-63, a human osteosarcoma cell line cell, was examined by MTT assay. Plumbagin caused a significant inhibition of MG-63 cell proliferation. Plumbagin exhibited high cytotoxicity against these cells in a dose-dependent manner with an IC<sub>50</sub> of 15.9 μg/mL. Overall, plumbagin showed potent anti-proliferative effect against these cells.

Effects of plumbagin on cell cycle distribution and cell morphology

Further to analyze the if plumbagin-induced growth inhibition of OS cells was a result of
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induction of apoptosis and/or cell cycle arrest, flow cytometric analysis was performed to assess the cell-population at various stages of cell cycle. Plumbagin induced apoptosis was investigated by PI staining. The results of cell cycle distribution analysis revealed the exposure of MG-63 cells to plumbagin, caused significant accumulation of cells in S-phase in a dose-dependent manner (Figure 3). Nearly 47% of the cells accumulated in S-phase as compared to 19.99% in control at 40 µg/mL of plumbagin. Furthermore, MG-63 cells treated with various concentrations of plumbagin for 72 h exhibited marked decrease in cell counts, cell shrinkage and loss of cell to cell contact (Figure 4). The effective suppression of cell

Figure 3. Effects of plumbagin on cell cycle distribution as examined by flow cytometric analysis. Cells were treated with plumbagin for 24 h.
周期性进展在癌症细胞中是一个有效的策略来阻止肿瘤生长 [27]。研究已经报告了化疗的和化疗预防剂诱导的细胞自杀或细胞周期停滞的癌症细胞在G0/G1或G2/M期 [28]。随后，涉及细胞周期调控和G1/S和G2/M转换的分子机制已经广泛研究。因此，设计能够针对细胞周期网络中调节剂的药物是一个有效的癌症治疗策略。

在plumbagin对S期细胞周期调节蛋白和DNA损伤标记的影响

为了进一步阐明细胞周期停滞是否与细胞周期检查点蛋白的调节有关，我们研究了plumbagin对S期关键蛋白的影响。在eukaryotes中，细胞周期蛋白A（cyclin A）和依赖cyclin的激酶2（CDK2）以及它们的复杂体作为主要调节剂，在S期进展中起着关键作用。MG-63细胞在暴露于plumbagin 24小时后，表现出了cyclin A和CDK2表达量的剂量依赖性下降（图5A）。cyclin A-CDK2复合物的变化可能干扰了S期的细胞周期进程。这些观察进一步确认了plumbagin通过诱导S期停滞而抑制了MG-63细胞的生长。

细胞周期蛋白和DNA损伤标记的评估

此外，细胞周期蛋白和DNA损伤标记，如p53和histone H2AX的表达也通过western blot分析评估。p53是一种细胞周期检查点蛋白，通过保留细胞的遗传稳定性而响应DNA损伤 [29]。ATM、ATR和DNA-PK等主要分子传感器在DNA损伤响应中被激活。这些观察进一步确认了plumbagin通过诱导S期停滞而抑制了MG-63细胞的生长。

图4。细胞暴露于plumbagin后的形态变化，通过相差显微镜观察（200×放大倍数）。

Influence of plumbagin on the expression of S-phase cell cycle regulators and DNA damage markers

To further elucidate whether cell cycle arrest is associated with the regulation of cell cycle checkpoint proteins, the influence of plumbagin on the expression of S-phase regulatory proteins was examined.

Cell-cycle proteins as-cyclin A, cyclin-dependent kinases 2 (CDK2) and their complexes, act as primary regulators and play a central role in S-phase progression in eukaryotes. MG-63 cells exposed to plumbagin for 24 h exhibited a dose-dependent decrease in cyclin A and CDK2 expressions (Figure 5A). The changes in cyclin A-CDK2 complex may possibly disturb the cell cycle progression at the S-phase. These observations further confirm that plumbagin inhibited the growth of MG-63 cells through the induction of S-phase arrest.

Furthermore, the expressions of DNA damage markers-p53 and histone H2AX were also assessed by western blot analysis. The tumor suppressor, p53 is a cell cycle checkpoint protein that preserves the genetic stability by mediating either cell cycle arrest or apoptosis in response to DNA damage [29]. Also, the major molecular sensors, including ATM, ATR, and DNA-PK, are activated in response to DNA damage.
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Figure 5. A. Effects of plumbagin on the expression levels of cyclin A and CDK2. Cells were exposed to plumbagin with the indicated concentrations for 24 h. Equal loading of the proteins was confirmed by stripping the immunoblots and reprobing for β-actin. B. Effects of plumbagin on the expression levels of phosphorylated p53 and phosphorylated histone H2AX. Cells were exposed to plumbagin with the indicated concentrations for 24 h. Equal loading of proteins was confirmed by stripping the immunoblots and reprobing for β-actin. Changes in the levels of protein expression are shown as ratios of the selected groups. C. Down-regulation of c-myc induced by plumbagin in MG-63 cells as examined by western blotting. Cells were treated with plumbagin for 24 h. Equal loading of proteins was confirmed by stripping the immunoblots and reprobing for β-actin.

damage, accompanied by the activation of signaling molecules, leading to cell cycle arrest or apoptosis [30]. In our study we observed a marked elevation in the expressions of phosphorylated p53 and phosphorylated histone H2AX in a dose-dependent manner (Figure 5B) following plumbagin exposure.

It is known that the phosphorylation and dephosphorylation of Ser 139-histone H2AX, another DNA damage marker, is closely related to DNA damage [31]. As speculated, P-histone (Ser 15) was significantly up-regulated in MG-63 cells treated with plumbagin. The data thus strongly suggest that plumbagin triggers DNA damage response proteins.

The transcription factor c-myc, product of oncogene c-myc, controls a numerous cell functions as cell proliferation, cell cycle regulation, differentiation, sensitization of the cells to apoptotic stimuli, and genetic instability [32-34]. Dysfunction of c-myc has been detected widely in human cancers [14]. Further, the expression of c-myc is observed to be strongly associated with cell proliferation in several malignancies [11, 15-17] and is overexpressed in osteosarcoma, and is involved with invasion and metastasis [14, 32, 33]. Studies have reported that the down-regulation of c-myc expression enhances sensitivity to chemotherapeutic drugs as cisplatin [35, 36]. Thus the observed marked downregulation of c-myc following plumbagin exposure suggests that plumbagin also improves cancer cell response to chemotherapy and also sensitizes cells to apoptosis. The higher concentration of plumbagin was more effective in regulating the expression of c-myc (Figure 5C).

Conclusion

It was observed that plumbagin effectively suppressed cancer cell proliferation and induced apoptosis via the inhibition of c-myc and the cell cycle arrest at S-phase.

Disclosure of conflict of interest

None.
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