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Research Article
Apoptotic Effect of Arctigenin on Human Renal Cancer Cells by Arresting Cell Cycle and Down regulating P13k/Akt Pathway

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Abstract

Background and Objective: The activation of P13k/Akt pathway is a major mechanism which inhibits apoptosis and leads to human malignancies. Arctigenin, a flavonoid, has antioxidant property and induces apoptosis in variety of cancer cells. Therefore, this study investigated the growth inhibitory and apoptotic activity of arctigenin against 786-O renal cancer cell (RCC) line and their underlying signaling mechanism. **Materials and Methods:** Dose dependent cell viability and cytotoxicity assay of arctigenin on 786-O RCC line was performed using MTT and LDH assay, respectively. Further, arctigenin-induced cell cycle arrest and apoptotic cell death was evaluated using flow cytometry. In addition, the effect of arctigenin in regulating pro-apoptotic caspase cascade and PI3k/Akt pathway was analyzed using standard kits and Western blotting. **Results:** MTT and LDH assay results showed significant anti-proliferative effect of arctigenin on 786-O RCC line, dose dependently. Furthermore, flow cytometric data indicated cell cycle arrest within G2/M phase and activation of apoptosis in RCC after arctigenin treatment. The association of signaling molecules in inducing apoptosis was confirmed by the elevation of caspase-9 and caspase-3 levels that further downregulated PI3k/Akt signaling pathway, dose dependently. **Conclusion:** The arctigenin exerts cytotoxic activity on renal cancer cells and perform apoptotic activity by downregulating PI3k/Akt signaling pathway.

Key words: Renal cancer, signaling pathway, anti-proliferative, arctigenin, apoptosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Renal cancer ranks second among urological cancers worldwide. Also, it ranks sixth in total mortality cases caused by different cancers in Western countries, accounting for around 100,000 deaths each year[1-3]. Followed by the surgical removal of localized renal cancer, 30-40% patients tend to develop metastatic renal carcinoma[4]. The therapies for metastatic renal cancer known till now comprise of chemotherapy, immunotherapy and targeted therapy. However, the lack of responsiveness towards current available therapies imposes the demand for novel therapeutic molecules for RCC[5,6]. Natural products are among these novel therapeutic compounds which are being widely studied against various cancer cell lines[7].

Cancer prevention by consuming fruits, vegetables and herbs rich diet has been accounted since ages, owing to the presence of natural compounds such as polyphenols within them. Recently, arctigenin, a polyphenol, has been investigated for its biological effects such as anti-inflammatory, anti-oxidant, anti-viral and anti-cancer properties[8-13]. Arctigenin is widely present in plants of family Asteraceae, such as *Arctium lappa*, *Saussurea medusa* and *Bardanae fructus*, as a phenylpropanoid dibenzylbutyrolactone lignin[14]. Various *in vivo* and *in vitro* studies reported anti-proliferative activity of arctigenin on different cell lines such as colon cancer, hepatic cancer and pancreatic cancer by varying molecular phenomenon. For example, it was reported that arctigenin can inhibit unfolded protein response induced by glucose deprivation and cause cytotoxicity in colon cancer HT-29 xenografts[12]. Similarly, in a study, arctigenin promoted necrosis by inhibiting mitochondrial respiration in human adenocarcinoma cell A549[15]. Furthermore, cell cycle arrest in addition to apoptosis was also demonstrated by arctigenin on gastric[16], lung[17] and human breast cancer cells[18]. The most identified molecular mechanism contributing for anti-proliferative effect of arctigenin on various cancers is apoptosis. Cells maintain their cellular balance by apoptosis which upon disturbance causes uncontrolled cell growth[19]. Cell performs apoptosis by various pathways including phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway[20]. PI3K, a dimer enzyme, contributes to the cell proliferation and survival after cytokine activation followed by Akt mediated biological responses which is a downstream effector molecule of PI3K. Further, the downstream substrates of Akt contribute to the stimulation of cell survival signals either in cytoplasm or nucleus[21].

A large number of experimental studies have shown the anti-proliferative effect of arctigenin on prostate cancer cells and hepatic cancer cells, via, deactivating PI3K/Akt pathway[22,23]. Nevertheless, to author's knowledge, the anti-proliferative effect of arctigenin on renal cancer cell line has not been elucidated. Furthermore, the role of arctigenin in deactivating PI3K/Akt signaling pathway in renal cancer cells is still unidentified.

Henceforth, the present study aimed to reveal the anti-proliferative and apoptotic effect of arctigenin on renal cancer cells and to determine the apoptotic effect of arctigenin associated with deactivation of PI3K/Akt pathway in renal cancer cells.

MATERIALS AND METHODS

Chemicals and cell culture: This study was performed in Central Hospital of Minhang District, Shanghai, China, from 2016-2017. Arctigenin with >98% purity was purchased from Sigma-Aldrich (St. Louis, MO) and its stock solution (50 mM) was prepared in Dimethyl sulfoxide (DMSO) (Sigma). The stock solution of arctigenin was stored at -20°C till further use. The 786-O RCC cell line was procured from American type culture collection (ATCC CRL1932). For cell culturing 786-O RCC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) which was supplemented with 10% fetal bovine serum (FBS, Sigma), streptomycin (100 μg mL^{-1}, Lonza, Basel, Switzerland) and penicillin (100 U mL^{-1}, Lonza, Basel, Switzerland). Cells were incubated and cultured in a humidified chamber (95% air and 5% CO_2) at 37°C till cells were approximately 80% confluent. Monoclonal Antibodies (mAbs) were purchased from Santa Cruz biotechnology, inc.

MTT assay: In brief, after seeding 786-O cells for 12 h at 37°C in a 96 well plate, the cells were treated with arctigenin at varying doses (0, 0.5, 1, 2.5, 5 and 10 μM) and were further incubated till predetermined time points (0, 24, 48 and 72 h). MTT assay was performed using cell proliferation kit (Sigma) according to manufacturer's protocol. The number of viable cells, following treatment was calculated by measuring optical density at 570 nm wavelength using UV-Vis spectrophotometer. For accuracy, experiments were repeated in triplicate and expressed as Mean±SD.

Lactate Dehydrogenase (LDH) assay: LDH is an enzyme which is present in the cell cytoplasm and releases when the cell or cell membrane is damaged. Therefore, the presence

of LDH enzyme in the medium can be evaluated as a parameter of cell cytotoxicity. Here, the concentration of LDH enzyme was estimated by cytotoxicity detection kit (Sigma) in accordance to prescribed protocol. The level of LDH was evaluated at 490 nm wavelength using UV-Vis spectrophotometer. For accuracy, experiments were repeated in triplicate and expressed as Mean±SD.

Cell-cycle arrest evaluation using flow cytometer: To evaluate the cell cycle arrest, arctigenin was added to overnight incubated 786-O cells at different doses. After 48 h of treatment, the culture medium was centrifuged at 1000 rpm for 5 min to procure the cells as pellet. After trypsinization, 80% ice cold ethanol was added to fix the cells and washed with PBS two times. Subsequently, 1% v/v of triton X-100 was added and cells were incubated at 37°C for 20 min. The apoptotic rate and the cell number in different cell cycle stages were then evaluated using FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) after staining the cells by propidium iodide solution. Propidium iodide is membrane impermeant dye which stains the cellular DNA by intercalating between the double strands.

Evaluation of apoptotic cells using annexin V binding assay: The 786-O cells were overnight incubated in 6 well-plates and treated with arctigenin at predetermined doses for 48 h. After incubation, cells were collected and annexin V and propidium iodide (PI) staining was performed using FITC Annexin V apoptosis detection kit I (BD Pharmingen), as described in manufacturer's protocol. Analysis of apoptotic cells was done by flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA, USA) and post-compensation and analysis of FACS data was performed by flowing 2.5.1 version software.

Caspase activity measurement: Caspase-3 and caspase-9 levels were determined using fluorometric protease analysis kit according to manufacturer's protocol. Arctigenin treated 786-O cells (48 h) were collected as pellet and homogenized in ice cold lysis buffer, provided in the kit for 10 min at 4°C followed by centrifugation. Supernatant was then mixed with equal quantity of fluorogenic substrate for 1 h at 37°C. Caspase activity was measured at 400 nm in a microtiter plate reader which is equivalent to the released fluorogenic moiety.

Western blot analysis: Western blot analysis was performed as previously described with slight modification[24]. Briefly, 786-O cells were treated with arctigenin for 48 h and cells were further collected in ice cold Radioimmunoprecipitation Assay (RIPA) buffer having protease inhibitor cocktail (Sigma). Following centrifugation supernatant was collected and the concentration of protein was estimated using Bradford dye-binding protein assay (Thermo Fisher Scientific). Proteins were then separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS PAGE). Proteins bands were further transferred electrophoretically to polyvinylidene difluoride membrane and probed with primary mAbs Akt (11E7)/phospho-Akt (Ser473) mAb and PI3K p85 (19H8)/phospho-PI3K p85 (Tyr458) mAb. Membrane was further washed and incubated for 1 h with secondary antibody i.e. Horseradish Peroxidase (HRP) conjugated rabbit anti-mouse (Santa Cruz Biotech, CA, USA). Level of protein expression was monitored as bands by chemiluminescence detection system (GE healthcare life sciences).

Overexpression of Akt allele: Briefly, 3×10^4 786-O cells were first seeded in each well of 48-well plate and transfected with myr-Akt encoding myrAktdeltaPH plasmid DNA (0.75 µg, Addgene, Cambridge, MA:10841) for 8 h using transfection reagent (Thermo Fisher Scientific) in according to manufacturer's protocol. Empty plasmid pECE (Addgene:26453) was taken as control. Afterwards, arctigenin was treated to the cells for 24 h and MTT assay was conducted to assess the cell viability.

Statistical analysis: The data was statistically evaluated using two-tailed student t-test and was expressed as Mean±SD. Data value of $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Arctigenin affects cell viability of 786-O cells: The effect of arctigenin on the viability of 786-O RCC cells was evaluated by MTT assay every 24 h till 72 h post-treatment. As observed, there was a dose and time dependent effect of arctigenin on the viability of RCC cells (Fig. 1a). Although arctigenin showed highest growth inhibitory effect (~72%) at a dose of 10 µM after 72 h of treatment, ~35 and ~41% reduction in cell viability was observed as early as 24 h of treatment at 5 and 10 µM doses, respectively. After 72 h of treatment, arctigenin showed significant reduction in cell viability even at low doses. In agreement to MTT results, LDH assay also showed dose and time dependent cell cytotoxicity of arctigenin on 786-O RCC cell line (Fig. 1b).

Arctigenin arrest cell cycle and induce apoptotic cell death in 786-O cells: Figure 2a shows that there was dose dependent increase in arctigenin induced cell apoptosis after 48 h of treatment in 786-O cells. Highest apoptotic rate, i.e.

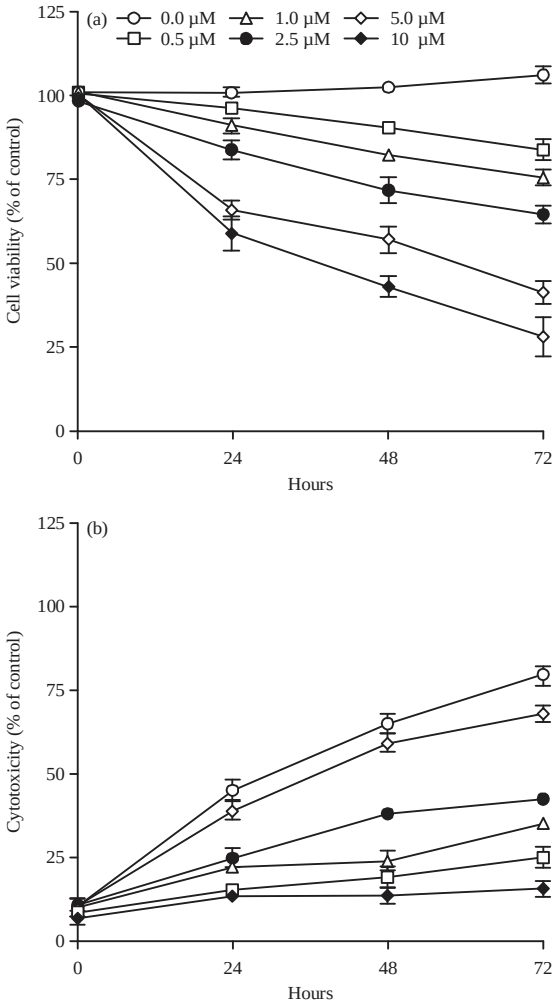

Fig. 1(a-b): Antiproliferative effect of arctigenin on 786-O renal cancer cell (RCC) line. (a) Dose dependent cell viability was determined by MTT assay and (b) Dose dependent cytotoxicity was performed by LDH assay

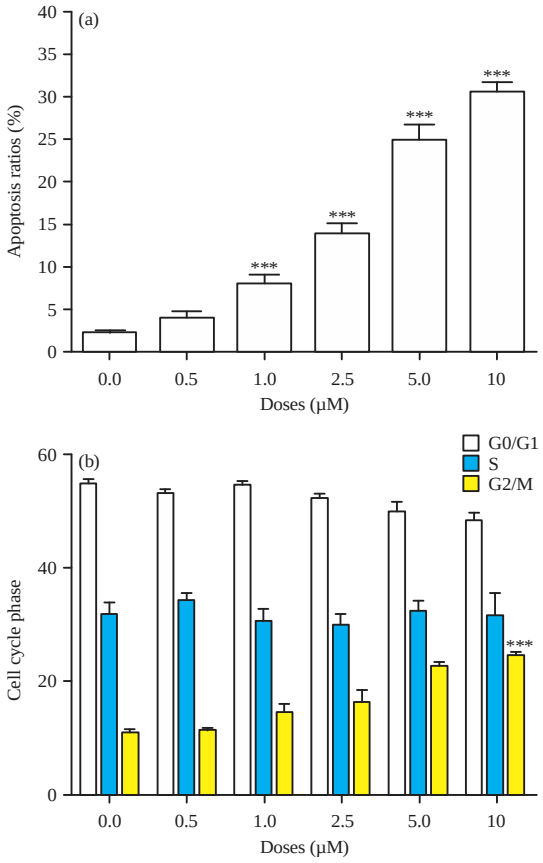

Fig. 2(a-b): Effect of arctigenin on apoptosis induction and cell cycle arrest in 786-O renal cancer cell (RCC) line (a) Arctigenin increases the apoptotic ratio of 786-O renal cancer cell (RCC) line in a dose-dependent manner and (b) Phases of cell cycle arrest in 786-O renal cancer cell (RCC) line
Data are shown as Mean±SD. The data was statistically evaluated using two-tailed student t-test and a value of $p<0.05$ was considered as significant (**$p<0.01$, ***$p<0.001$)

~30%, was observed at 10 µM dose. Also, significant apoptosis was also observed at 1, 2.5 and 5 µM. Additionally, 786-O cells showed that cell cycle was arrested at G2/M phase at10 µM dose which can be an indicative of ongoing apoptosis (Fig. 2b).

Arctigenin activates caspase cascade in 786-O cells: Caspase cascade is a decisive initiator of cell apoptosis[25]. Significant activation of caspase-3 and caspase-9 in 786-O cell was observed upon arctigenin treatment after 48 h, dose dependently as compared to control cells (Fig. 3a, b). At the maximum dose, i.e. 10 µM, increase in 57 and 72% was evaluated for caspase-3 and caspase-9, respectively.

Arctigenin inhibits PI3k/Akt activation: The association of PI3k/Akt signaling pathway with cell growth, differentiation, apoptosis, cell cycle regulation and tumorigenesis is well documented[26,21]. As shown in Fig. 4, arctigenin dose dependently inhibited phosphorylated PI3k (p-PI3k) and phosphorylated-Akt (p-Akt) levels in 786-O cells. Further, Akt was over expressed by transfecting the cells with myrAktdeltaPH plasmid DNA to validate the role of PI3k/Akt signaling pathway in inducing cell apoptosis. Figure 5 shows that myrAktdeltaPH plasmid DNA increased the cell viability in their respective treatment group depicting that arctigenin downregulate PI3k/Akt pathway in 786-O cells leading to apoptosis.

Previous studies showed that the mechanism of cytotoxicity of arctigenin varies among tissues and cell

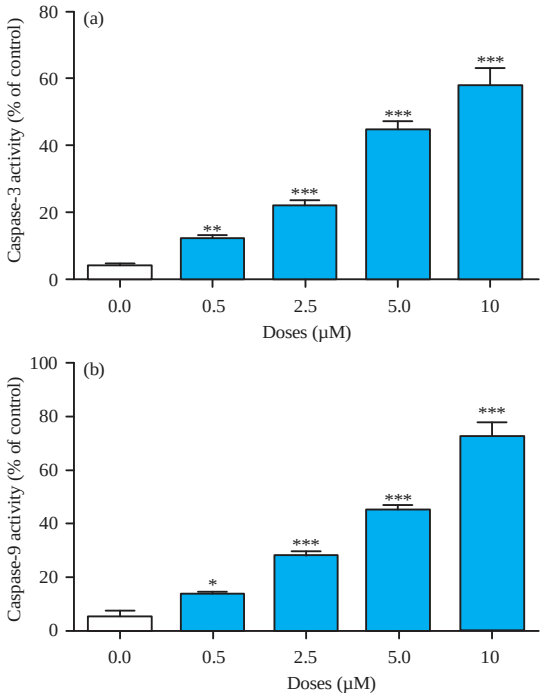

Fig. 3(a-b): Influence of arctigenin on (a) caspase-3 and (b) caspase-9 activities.

Data are shown as Mean±SD. The data was statistically evaluated using two-tailed student t-test and a value of p<0.05 was considered as significant (*p<0.05, **p<0.01, ***p<0.001)

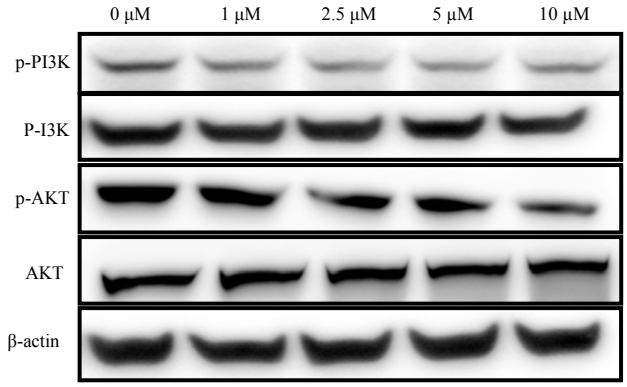

Fig. 4: p-PI3K, PI3K, p-Akt, Akt and β-actin expression in arctigenin treated 786-O renal cancer cell (RCC) line

types[12,15]. In this study, the PI3k/Akt pathway mediated chemopreventive effect of arctigenin on RCC 786-O cell line has been demonstrated. Initially, MTT and LDH assay were performed to observe the cytotoxic effect of arctigenin at 0, 0.5, 1.0, 2.5, 5.0 and 10 μM doses till 72 h. It was observed that arctigenin exerted antiproliferative effect on 786-O cells in a dose and time dependent manner (Fig. 1). As previously studied, arctigenin exerts antiproliferative effect due to

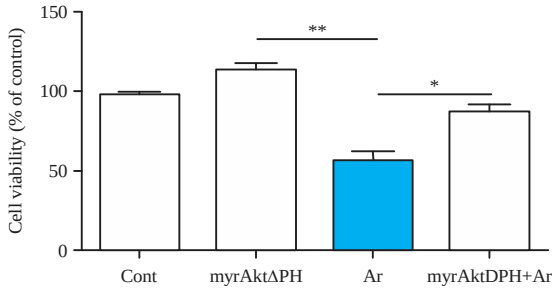

Fig. 5: Arctigenin induced antiproliferative effect on 786-O renal cancer cell (RCC) line after overexpression of Akt allele

Data are shown as Mean±SD. The data was statistically evaluated using two-tailed student t-test and a value of p<0.05 was considered as significant (*p<0.05, **p<0.01)

apoptosis and performs cell cycle arrest on various cancer cells[27,28], the apoptotic effect of arctigenin on 786-O cells was investigated. Flow cytometry results revealed that arctigenin significantly inhibits cell cycle progression in 786-O cells at and above 1 μM dose after 48 h of treatment (Fig. 2).

It is well accepted that the progression of cancer is mainly due to dysregulation of cell cycle. In mammalian cells, there are four phases in a cell cycle (G1, S, G2 and M) which are regulated by checkpoints. These checkpoints confirm the proper accomplishment of previous cell phase processes, such as proper DNA replication and formation of chromosome-spindle attachment, before entering into subsequent phase. Dysregulation of G1/S or G2/M checkpoints leads to uncontrolled cell growth and results in the conversion of normal cells into cancerous cancer[29]. Although, previous reports observed that arctigenin can induce cell cycle arrest in G_0/G_1 phase in glioma cells[22], the present study clearly indicates that arctigenin treatment results in cell cycle arrest at G2/M phase as reported by Yoo *et al.*[30], in SW480 colon cancer cell line.

Apoptosis is initiated by either extrinsic or intrinsic apoptotic pathway. Extrinsic pathway responds to extracellular stimuli and involves death receptors while intrinsic pathway is modulated by mitochondrial factors within the cell[31]. After receiving stress signals from various signal transduction cascades, mitochondria releases cytochrome-c which activates the initiator caspase of the caspase cascade, i.e. caspase-9 and initiates apoptosis. The involvement of both extrinsic and intrinsic apoptotic pathways has been demonstrated in hepatocellular carcinoma cells after arctigenin treatment[23]. In the present study, increase in caspase-9 and caspase-3 in 786-O renal cancer cells upon arctigenin treatment showed induction of apoptosis (Fig. 3). Arctigenin has also been shown to induce necrosis in other cell line[15].

Literature survey suggested that phosphatidylinositol plays an important role as a secondary messenger in signaling pathways which regulates downstream cellular processes like cell growth, cell differentiation and apoptosis. Activation of PI3k (a dimeric enzyme) results in the phosphorylation of phosphatidylinositol, activating downstream targets such as Akt and acts as survival signal. PI3k is composed of two subunits i.e., regulatory unit (p85) and catalytic unit (p110). The catalytic unit is accountable for Akt phosphorylation and activation which in turn phosphorylates and downregulates pro-apoptotic proteins, such as caspase-9, along with upregulation of anti-apoptotic proteins, such as Bcl-2, inhibiting apoptosis[32]. It has previously suggested that flavonoids competes with ATP binding sites and inactivates phosphorylation of signaling proteins[33]. Lu *et al.*[23] observed that arctigenin deactivates P13k/Akt pathway and induces apoptosis in human hepatocellular carcinoma cell lines HepG2 and SMMC7721. Therefore, P13k/Akt is becoming a target signaling pathway in the search for novel anticancer agents such as arctigenin[34]. Current data also showed arctigenin indeed inhibits PI3k/Akt signaling pathway in 786-O RCC cell lines, as indicated by reduced level of p-PI3k and p-Akt (Fig. 4), which were further restored by overexpression of Akt (Fig. 5).

Although, natural products from plants are considered as effective and safe chemopreventive agents, their clinical translation is limited due to low bioavailability. Thus, chemopreventive effect of arctigenin, *in vivo,* need to be analyzed for its future use in clinics and studies to enhance its bioavailability are also prerequisite.

CONCLUSION

One of the effective cancer prevention and treatment therapy consists of the use of natural phytochemicals with minimal side effects. Till date, various studies showed numerous natural products which can regulate the cell signaling mechanisms and are capable to induce apoptosis in cancer cells. Current study observed the cytotoxic and apoptotic role of arctigenin against 786-O renal cancer cells progression by downregulating PI3k/Akt signaling pathway.

SIGNIFICANCE STATEMENT

This study showed that arctigenin, a natural polyphenol, can be used as a therapeutic and protective molecule against renal cancer initiation. Moreover, this study provides a future direction for medical and pharmaceutical applications of arctigenin in improvising current therapies used in treatment of renal cancer.

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