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Plumbagin induces cell death through a copper-redox cycle mechanism in human cancer cells

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Plumbagin, a naphthoquinone derived from the medicinal plant *Plumbago zeylanica* has been shown to exert anticancer and anti-proliferative activities in cells in culture as well as animal tumor models. In our previous paper, we have reported the cytotoxic action of plumbagin in plasmid pBR322 DNA as well as human peripheral blood lymphocytes through a redox mechanism involving copper. Copper has been shown to be capable of mediating the action of several plant-derived compounds through production of reactive oxygen species (ROS). The objective of the present study was to determine whether plumbagin induces apoptosis in human cancer cells through the same mechanism which we proposed earlier. Using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay, 3-(4,5-B-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell growth inhibition, histone/DNA ELISA, homogeneous caspase-3/7 assay for apoptosis as well as alkaline comet assay for DNA single-strand breaks detection in this report, we confirm that plumbagin causes effective cell growth inhibition, induces apoptosis and generates single-strand breaks in cancer cells. Incubation of cancer cells with scavengers of ROS and neocuproine inhibited the cytotoxic action of plumbagin proving that generation of ROS and Cu(I) are the critical mediators in plumbagin-induced cell growth inhibition. This study is the first to investigate the copper-mediated anticancer mechanism of plumbagin in human cancer cells and these properties of plumbagin could be further explored for the development of anticancer agents with higher therapeutic indices, especially for skin cancer.

Introduction

Several methods exist for the treatment of cancer in modern medicine including chemotherapy, radiotherapy and surgery. However, with persistent failure of existing regimens, it is highly desirable to find novel agents for the treatment of cancers. In search of such agents, studies have been focused on naturally occurring chemical compounds as several among them are known to possess cytotoxic effects and have the potential for killing cancer cells (1).

Plumbagin isolated from the roots of *Plumbago zeylanica L.* has been proven to possess anti-tumor activity both *in vitro* and in *in vivo* studies. It has been found to inhibit the growth of Raji, Calu-1, HeLa and Wish cell lines (2). Recent studies indicate that plumbagin can down-regulate the expression of nuclear factor kappa B (NF-κB) regulated gene products involved in the cell proliferation and anti-apoptosis. Inhibition of the NF-κB activation pathway by plumbagin was found to increase the apoptotic activity of tumor necrosis factor and paclitaxel (3). Interestingly in a very recent report, plumbagin was shown to induce cell cycle arrest and apoptosis through reactive oxygen species (ROS) in human melanoma A375.S2 cells (4). Although a natural agent, plumbagin has some toxicity associated with it that has been extensively evaluated in rodents. Toxic side effects included diarrhea, skin rashes and hepatic and reproductive toxicity (3). These toxic side effects were dose related. The LD_{50} for these side effects in mice was 8–65 mg/kg body weight for oral (p.o.) administration and 16 mg/kg body weight for intra-peritoneal (i.p.) (3). Yet plumbagin has been reported to be nontoxic at doses (2 mg/kg body weight i.p. or 200 ppm in diet) shown to elicit therapeutic effects (3).

Even though there are reports of plumbagin having anti-tumor activity, there are only very few studies on the mechanism of cell death induced by plumbagin in human cancer cells. So elucidation of the mechanisms by which plumbagin induces its anticancer therapeutic effect is necessary to provide a solid foundation for its use as an agent for prevention strategies. Earlier studies have established that several compounds of plant origin such as flavanoids, tannins, curcumin and the stilbene–resveratrol are themselves capable of inducing oxidative DNA damage either alone or in the presence of certain transition metal ions especially copper (5–8). Copper is an essential trace element which is distributed throughout the body (9). Besides forming the essential redox-active centre in a variety of metalloproteins, copper has also been found in the nucleus and to be closely associated with the chromosomes and DNA bases, particularly guanine (10). DNA-associated copper has been suggested to be involved in maintaining normal chromosome structure and in gene regulatory processes (11–13). The concentration of copper in blood is $\sim$16 μM (14). Further diet-derived copper enters the liver preferentially in the monovalent Cu(I) form (15). Interestingly, this ion has been found at higher concentrations in breast tumors as compared with the normal breast tissue (9). Further, it has also been shown that $\sim$20% of cellular copper is present in the nucleus (16), associated more specifically with DNA bases particularly guanine (10). Studies have also shown that metals, particularly copper, are capable of mediating the activation of several compounds, such as benzoyl peroxide, quercetin and dietary flavanoids by a redox mechanism leading to the formation of reactive oxygen and other organic radicals

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(5,17). It was also previously shown that copper can induce the oxidation of 1,4 HQ, a benzene metabolite, producing 1,4 BQ and H_2O_2 through a semiquinone intermediate (18). Many clinically important anti-tumor drugs contain quinone nucleus (19). The anti-tumor and cytotoxic effects of quinoid drugs are thought to be mediated through their one electron reduction to semiquinone radicals (20). In our earlier report, we for the first time confirmed using plasmid DNA and also in a cellular system of lymphocytes isolated from human peripheral blood, the role of metal ion (copper) in the observed cytotoxic action of plumbagin (21), which possibly involves mobilization of endogenous copper ions and the subsequent pro-oxidant action. In this communication, we report the cytotoxic action of plumbagin in human cancer cells through a redox cycle mechanism involving mobilization of endogenous copper and the consequent pro-oxidant action.

Material and methods

Materials

Plumbagin used in the experiments was isolated from the hairy root cultures established in our laboratory from the medicinal plant *P. zeylanica*. The purity and identity of plumbagin were fully established by chromatographic methods, UV, IR, NMR and HPLC. The sample of plumbagin was identical with an authenticated sample. Stock concentrations of drugs were prepared in dimethyl sulphoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM), DMSO, ethidium bromide, acridine orange and 3-(4,5-B-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St Louis, MO). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was obtained from Promega Co. (Madison). All other chemicals were of analytical grade.

Cell line, culture and treatments

Human skin carcinoma A-431 cell line was procured from National Center for Cell Science (Pune, India) and maintained at Laboratory of Tumor Immunology and Functional Genomics, Regional Cancer Centre (Thiruvananthapuram, India). SKBR3 cells were obtained from American Type Culture Collection. The cells were maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% foetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. In order to test cell growth inhibitory and apoptotic effects of plumbagin in unrelated cancer cell lines, varying exposure time points and concentrations were used.

Cell viability assay

Inhibition of cell proliferation by plumbagin was measured by MTS assay. A-431 cells (5000 cells/well), control 1 (medium only) and control 2 (medium + cells) were seeded in triplicates in 96-well microtitre plates and incubated for attachment at 37°C in 5% CO_2 incubator for 12 h. After 12 h, 100 μl of different concentrations of plumbagin (concentration ranging from 1 to 100 μM) were added to each well excluding the control wells. The plates were then incubated at 37°C in 5% CO_2 incubator for 24–48 h. In all, 20 μl of MTS–PMS solution was added and incubated in dark for another 4 h and absorbances were recorded at 490 nm using ELISA plate reader. Percentage cell viability was calculated using the formula of Kumi-Diaka *et al.* (22). For SKBR3 cells, growth inhibition was assayed using MTT assay. Cells were exposed to different concentrations of plumbagin (0–5 μM). After the incubation, 20 μl of the MTT dye was added [5 mg/ml in phosphate-buffered saline (PBS)] to each well and the plates were incubated further for 2 h. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in 100 μl of isopropanol. The plates were gently rocked for 30 min on a gyratory shaker, and absorbance was measured at 595 nm using ULTRA Multifunctional Microplate Reader (Tecan, Durham, NC).

Deducing cell morphology by acridine orange/ethidium bromide dual stain, histone/DNA ELISA assay and homogenous caspase assay (apoptosis analysis)

Drug-induced apoptosis and necrosis were determined morphologically after labeling with acridine orange and ethidium bromide (23). A-431 cells (5000 cells in 100 μl) were cultured with 100 μl of different concentrations of plumbagin at 37°C in 5% CO_2 incubator for 12–48 h. Then the medium was removed and 1 μl of 100 μg/ml of acridine orange with 100 μg/ml of ethidium bromide was added to cells. After a few minutes, the cells were immediately washed with PBS once and viewed under fluorescent microscope with a suitable filter. Nucleosomal DNA was assayed with the ELISA^{PLUS} (Roche, Indianapolis, IN) kit using the protocol provided by the manufacturer with minor modifications. SKBR3 cells were plated in duplicate wells at 2.0×10^5 per well in six-well dishes and cultured overnight. Plumbagin was added at 0–5 μmol/litre. At 72 h, both floating and trypsinized cells were collected and washed and both viable and dead cells were counted by trypan staining. In another set of experiments, 50 000 cells (live or dead) were extracted with 500 μl incubation buffer, and after centrifugation, the supernatant was saved at −20°C. A sample of 100 μl of each extract (diluted 1:10 in incubation buffer) was processed per protocol. Signal was assayed for absorption at 405 nm in the Ultra plate reader. The mean and standard deviation from at least two independent experiments (*n* of at least 4) were plotted with Prism software. Caspase-3/7 homogeneous assay was performed using a kit purchased from Promega Co. and the protocol provided by the manufacturer. Cells were treated with plumbagin (0–5 μM/litre) or DMSO control for 72 h. After treatment, 100 μl Apo-ONE caspase-3/7 reagent was added and plates were shaken for 2 min, followed by incubation at room temperature for 3 h. The fluorescence was then evaluated using ULTRA Multifunctional Microplate Reader (Tecan) at excitation/emission wavelengths of 485/530 nm.

Determination of ROS generation and Cu(II)/Cu(I) redox cycling

To investigate the mechanism of plumbagin-mediated cell growth inhibition and also to demonstrate whether ROS generation and Cu(II)/Cu(I) redox cycling are involved in plumbagin-induced cell death in cancer cells, the free radical scavenging agents [superoxide dismutase (SOD), catalase and thiourea] and Cu(I)-specific sequestering agent (neocuproine) were used in this study. The A-431 cells seeded in 96-well plate were pre-treated with SOD, catalase, thiourea and neocuproine for 1.5 h and, subsequently, treated with plumbagin (25 μM). One set of cells was treated with plumbagin (25 μM) only. The plates were then incubated at 37°C in 5% CO_2 incubator for 24 h; 20 μl of MTS–PMS solution was added and incubated in dark for another 4 h and absorbances were recorded at 490 nm using ELISA plate reader.

Isolation of lymphocytes

Heparinized blood samples (2 ml) from healthy donors were obtained by venepuncture and diluted suitably in Ca^{++}- and Mg^{++}-free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma Chemical) and the cells ($\approx 2 \times 10^5$) were suspended in RPMI 1640.

Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using trypan blue exclusion test (24). The viability of the cells was found to be >93%.

Treatment of lymphocytes

Lymphocyte DNA breakage was studied after pre-incubating the cells with various concentrations of plumbagin after which the cells were washed twice with PBS and incubated further in the presence of $CuCl_2$. In another set of experiments, lymphocytes were pre-incubated with 20 μM Cu(II) prior to incubation with plumbagin. After the incubation, the reaction mixture was centrifuged at 4000 r.p.m., the supernatant was discarded and pelleted lymphocytes were re-suspended in 100 μl of PBS and processed further for comet assay.

Comet assay

Previously, our group has shown that mobilization of endogenous copper and the consequent oxidative DNA damage caused by plant-derived compounds results in DNA single-strand breaks (8). The alkaline version of comet assay is a sensitive technique to identify DNA single-strand breaks. Therefore, comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* (1998) (25) with slight modifications. Fully frosted microscope slides pre-coated with 1.0% normal melting agarose at ~50°C (dissolved in Ca^{++}- and Mg^{++}-free PBS) were used. Around 10 000 cells were mixed with 75 μl of 1.0% low melting point agarose (LMPA) to form a cell suspension and pipetted over the first layer and covered immediately by a cover slip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The cover slips were removed and third layer of 0.5% LMPA (75 μl) was pipetted and cover slips were placed over it and allowed to solidify on ice for 5 min. The cover slips were removed and slides were immersed in cold lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 and 1% Triton X-100 added just prior to use for minimum of 1 h at 4°C. After lysis, DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH >13. Electrophoresis was performed at 4°C in field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 μl ethidium bromide (20 μg/ml) and covered with a cover slip; the slides were placed in a humidified chamber to prevent drying of the gel and analyzed the

same day. Slides were scored using an image analysis system (Komet 5.5; Kinetic imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510- to 560-nm excitation and 590-nm barrier filters) integrated CC camera; comets were scored at ×100 magnification. Image from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Komet 5.5 image analysis system.

Statistics

The statistical analysis was performed as described by Tice *et al*. (2000) (26) and is expressed as ±SEM of three experiments. A Student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed. *P*-values < 0.05 were considered statistically significant.

Results

Plumbagin inhibits growth of A-431 cells

In order to verify cell growth inhibition of A-431 cells by plumbagin, MTS assay was performed. As can be seen from results of Figure 1A, plumbagin caused a clear concentration-dependent inhibition of growth of the human skin carcinoma (A-431) cell line (IC50 25 μM). This inference is of greater significance as plumbagin has been shown to exert anti-proliferative effect on skin cancer in various reports (7,27). In order to confirm that the plumbagin-mediated cell growth inhibition is not cell type specific, we tested plumbagin on an unrelated breast cancer cell line SKBR3. Results of MTT assay reveal that incubation of SKBR3 cells with increasing doses of plumbagin lead to a progressive inhibition of cell growth (Figure 1B).

Plumbagin induces morphological alterations (characteristics of apoptosis) in human skin carcinoma A-431 cells

The phenotypic characteristics of plumbagin-treated cells were evaluated by microscopic inspection of overall morphology. Treatment of cells with the indicated concentration of plumbagin for 6 h resulted in nuclear condensation, which was clearly evident in the light microscopy. When cells were treated at same concentrations for up to 12 h or more, cells formed many apoptotic bodies. In contrast, normal untreated cells were well spread with normal morphology. A-431 cells untreated after 12, 18 and 24 h showed the green fluorescence representing 100% viability (Figure 2A). A-431 cells treated with 25 μM of plumbagin showed varying degrees of evident apoptosis in a time-dependent manner. Nuclear and cytoplasmic condensation with blebbing of the plasma membrane, and formation of apoptotic bodies (orange-red fluorescence) were prominent at the end of 12, 24 and 48 h, the latter being severe (Figure 2B, C and D). However, some cells showed necrosis (red fluorescence) as the membrane integrity was lost due to cell rupture releasing noxious cellular contents (Figure 2D). To further confirm that plumbagin could induce apoptosis in different cancer cell lines histone/DNA ELISA assay was performed. This is a sensitive assay to detect apoptosis in whole cells. As can be seen from results of Figure 2E, exposure of SKBR3 cells to plumbagin (0–5 μM) lead to a progressive increase in histone-reactive material as indicated by increase in optical density at 405 nm. Finally, homogenous caspase assay was performed to further confirm the induction of apoptosis by plumbagin in SKBR3 cells. Results of Figure 2F clearly show a progressive increase in caspase-3/7 relative fluorescence units upon exposure to plumbagin (0–5 μM for 72 h).

Free radical scavengers and neocuproine (a Cu(I)-specific sequestering agent) show preventive effect on plumbagin-induced apoptotic response in A-431 cells

To investigate the mechanisms leading to loss of cell proliferation by plumbagin and also to demonstrate whether ROS generation and Cu(II)/Cu(I) redox cycling are involved in plumbagin-induced cell death in cancer cells, various free radical scavenging agents (SOD, catalase, thiourea) and Cu(I)-specific sequestering agent (neocuproine) were used in this study. In the absence of plumbagin, free radical scavengers (SOD, catalase and thiourea) and neocuproine did not show any appreciable toxicity to the cells at the given concentration for the entire time period. Therefore, prior treatment of the cells with SOD, catalase, thiourea and neocuproine for 1.5 h followed by plumbagin treatment at indicated concentrations for 24 h was performed. Figure 3 shows that plumbagin-induced growth inhibition of A-431 cells can be inhibited by ROS scavengers and also neocuproine to a significant level, suggesting the involvement of ROS and a possible involvement of copper in the observed cytotoxic action of plumbagin. These results are in further support of our earlier study where it was shown that scavengers of ROS and neocuproine significantly inhibited the DNA breakage induced by plumbagin in a cellular system of lymphocytes isolated from human peripheral blood using comet assay. So these results strongly suggest that anticancer action of plumbagin may possibly be due to the mobilization of endogenous copper ions and the subsequent pro-oxidant action.

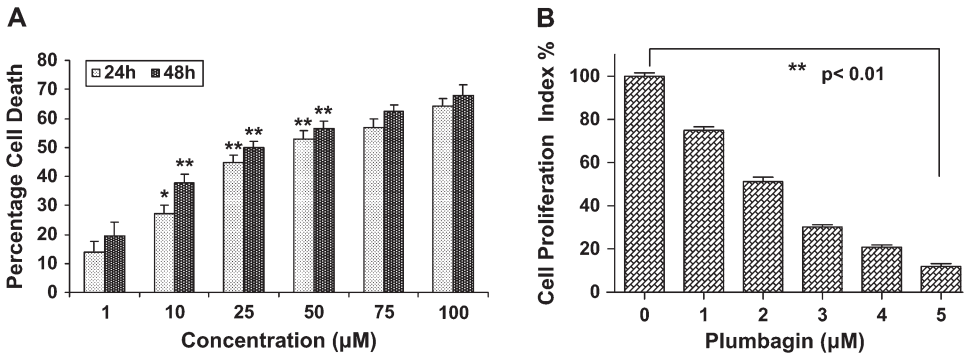

Fig. 1. (**A**) The effect of plumbagin on cell proliferation inhibition in A-431 cells by MTS assay. A-431 cells grown in 96-well plates were treated with indicated concentrations of plumbagin for 24–48 h. At the end of treatment, percentage cell death was assessed by MTS assay with triplicate samples as described in the Materials and Methods. '**' Represents $P < 0.01$ when compared to untreated control. (**B**) The effect of plumbagin on cell growth in SKBR3 breast cancer cells as detected by MTT assay. SKBR3 cells were incubated with increasing concentrations of plumbagin (0–5 μM) for 72 h in 96-well plates. MTT assay was performed as described under the Materials and Methods. All results are expressed as the mean percentage of control ± standard deviation of triplicate determinations from three independent experiments. '**' Indicates *P*-values < 0.01 when compared to untreated control.

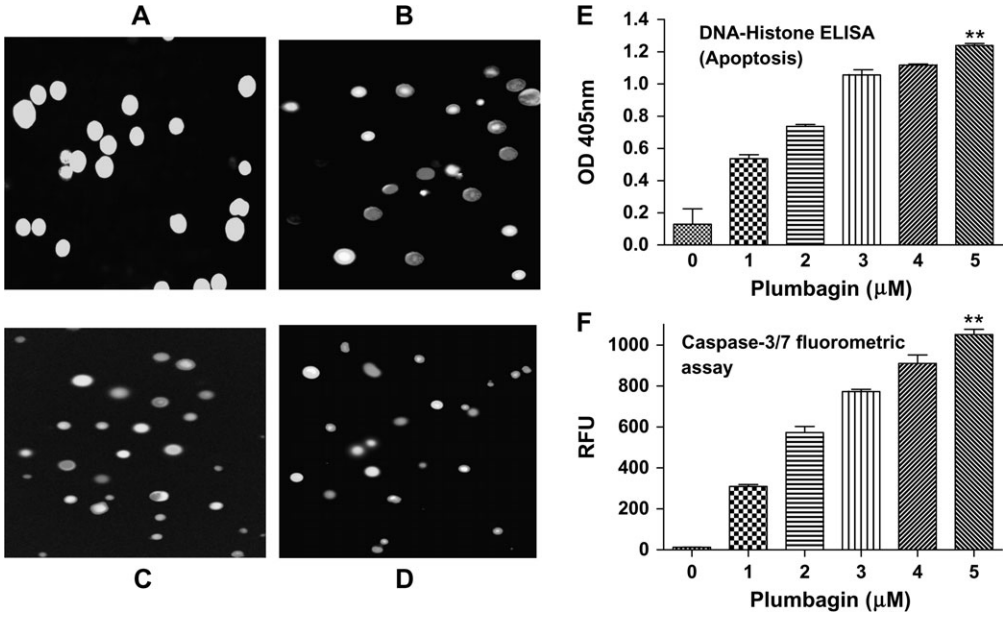

Fig. 2. Changes in nuclear morphology of A-431 cells induced by plumbagin. A-431 cells (5000 cells/well) were seeded in 12-well plates and then treated with or without plumbagin (25 μM) for different time periods. After washing with PBS, the cells were stained with a mixture of acridine orange–ethidium bromide mixture. The cells were viewed under inverted fluorescent microscope and photographed as described under the Materials and Methods. The experiment was repeated two times with similar results. (**A**) Control (untreated cells); (**B**) cells treated with plumbagin after 12 h; (**C**) cells treated with plumbagin after 24 h; (**D**) Cells treated with plumbagin after 48 h. (**E**) Nucleosomal DNA was assayed with the ELISA^{PLUS} (Roche) kit using the protocol provided by the manufacturer with minor modifications. SKBR3 cells were plated in duplicate wells at 2.0×10^5 per well in six-well dishes and cultured overnight. Cells were exposed to plumbagin (0–5 μmol/litre). At 72 h, both floating and trypsinized cells were collected and washed and both viable and dead cells were counted by trypan staining. Fifty thousand cells (live or dead) were extracted with 500-μl incubation buffer, and after centrifugation, the supernatant was saved at −20°C. A sample of 100 μl of each extract (diluted 1:10 in incubation buffer) was processed per protocol. Signal was assayed for absorption at 405 nm in the Ultra plate reader. $^{**}P < 0.01$ compared to untreated control. (**F**) Homogeneous caspase-3/7 assay for apoptosis: caspase-3/7 homogeneous assay was performed using a kit purchased from Promega Co. (Madison, WI) and the protocol provided by the manufacturer. Cells were treated with plumbagin (0–5 μM/litre) or DMSO control for 72 h. After treatment, 100 μl Apo-ONE caspase-3/7 reagent was added and plates were shaken for 2 min, followed by incubation at room temperature for 3 h. The fluorescence was then evaluated using ULTRA Multifunctional Microplate Reader (Tecan) at excitation/emission wavelengths of 485/530 nm. $^{**}P < 0.01$ when compared to untreated control.

Effect of pre-incubation of lymphocytes with plumbagin or Cu(II) on plumbagin–Cu(II)-mediated DNA breakage as measured by comet assay

In a previous study, we had shown that increasing concentrations of plumbagin (10–50 μM) in the presence of 20 μM $CuCl_2$ induces extensive DNA breakage in isolated lymphocytes (21). Plumbagin alone at any of the concentrations tested did not damage lymphocyte DNA, whereas on additions of Cu(II), DNA damage to varying degrees was observed (21). In this report, we studied the lymphocyte DNA breakage after pre-incubating the cells with plumbagin and incubated further in the presence of $CuCl_2$. Results of Figure 4 indicate that, with increasing concentration of plumbagin, a progressive increase in DNA breakage [as indicated by increase in tail length (μm)] was observed. These results indicate that both plumbagin and Cu(II) are either able to enter the cells or bind to the cell membrane and, moreover, these results are in further support of our earlier findings that plumbagin–Cu(II) system is capable of DNA breakage in lymphocytes.

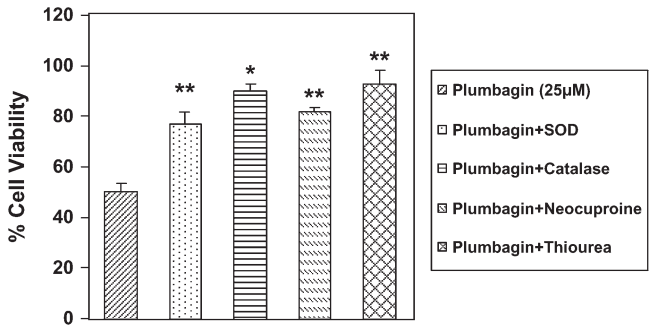

Fig. 3. Effects of free radical scavengers and neocuproine (a Cu(I)-specific sequestering agent) on plumbagin-induced cell death in A-431 cells. A-431 cells were plated into 96-well plate for 24 h and then treated with SOD (100 μg/ml), catalase (100 μg/ml), thiourea (1 mM) and neocuproine (1 mM) for 1.5 h followed by plumbagin (25 μM) treatment for a further 12 h. MTS was added to the medium for additional 3 h. The cell viability was expressed as percentage over the control. Each value is presented as the mean ± SEM of triplicate determinations from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, the mean percentage viability was significantly higher from the corresponding plumbagin-treated groups as analyzed by Student's *t*-test.

Discussion

Based on our own observations and those of others in literature, we have earlier proposed a mechanism for the cytotoxic action of plumbagin that involves mobilization of endogenous copper ions and the consequent DNA degradation through the generation of ROS. In the present study, to our knowledge, we demonstrated for the first time that plumbagin-mediated cell growth inhibition could be reversed by scavengers of ROS. Inhibition of cytotoxicity of plumbagin upon addition of neocuproine, a Cu(I)-specific sequestering agent, suggests the possible involvement of copper ions. These results corroborate our earlier results, where it was shown that plumbagin in the presence of micromolar concentrations of Cu(II) causes DNA damage in

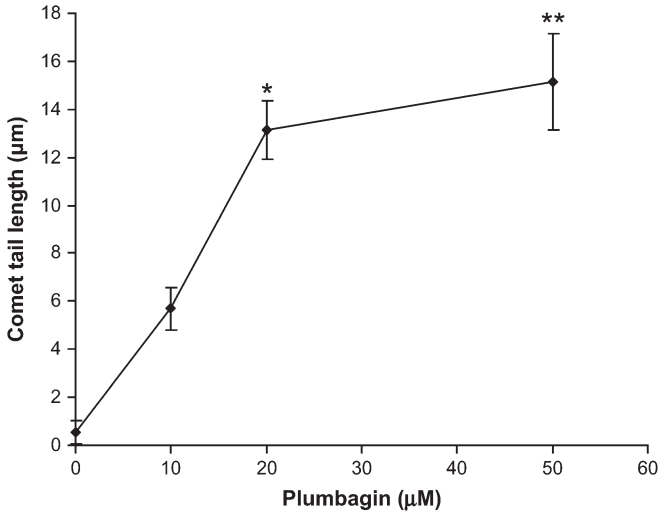

Fig. 4. Effect of pre-incubating the lymphocytes with increasing concentrations of plumbagin (0–50 μM) followed by Cu(II) (20 μM) on DNA breakage expressed as comet tail length. Values reported are ±SEM of three independent experiments. $^{**}P$-value < 0.01 and significant when compared to control.

plasmid pBR322 DNA and also human peripheral lymphocytes. Furthermore, in this study, we demonstrated that the pre-incubation of human peripheral lymphocytes with plumbagin or copper causes extensive DNA strand breaks in lymphocytes, which indicates that both plumbagin and Cu(II) are capable of either traversing the cell membrane or binding to it.

A very recent report established that ROS accumulation contributes to plumbagin-induced cell death in human melanoma cells (7). Enhancement of ROS production has long been associated with the apoptotic response induced by several anticancer agents (28,29). The generation of hydroxyl radicals in the proximity of DNA is well established as a cause of strand scission. It is generally recognized that such reaction with DNA is preceded by the association of a ligand with DNA followed by the formation of hydroxyl radicals at that site. Among the oxygen radicals, the hydroxyl radical is the most electrophilic with high reactivity and, therefore, possesses a small diffusion radius. Thus, in order to cleave DNA, it must be produced in the vicinity of DNA (30). The location of redox-active metals is of utmost importance for the ultimate effect because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (31). Moreover, the generation of hydroxyl radicals from the reaction of Cu(II) complexes with biological reductants such as ascorbic acid, glutathione, acetylcysteine and hydroquinone was already established by spin trapping experiments using electron spin resonance (32). Indeed, it was earlier proved that flavanoids are able to form a ternary complex with DNA and Cu(II), where Cu(II) is reduced to Cu(I) (33). Superoxide can also be formed by re-oxidation of Cu(I) to Cu(II) in the ternary complex. Chromatin-bound copper is understood to be present in the reduced form (Cu(I)) (34) and, thus, would be available for re-oxidation to Cu(II) by H_2O_2 in the Fenton-type reaction and binding to phenolic compounds and recycling.

These other observations in literature include the fact that copper along with zinc is the major metal ion present in the nucleus (35). The concentration of copper in various tissue ranges from 10 to >100 μM with 20% found in the nucleus (9). Further, serum (36), tissue (37) and cellular (38) concentrations of copper are greatly increased in various malignancies. Copper ions from chromatin can be mobilized by metal chelating agents giving rise to internucleosomal DNA fragmentation, a hallmark property of cells undergoing apoptosis (39). Further, it has also been proposed that most clinically used anticancer drugs can activate the late events of apoptosis (DNA degradation and morphological changes) and the essential signaling pathways differ between pharmacological cell death and physiological induction of cell death (40).

The issue of plumbagin bioavailability in *in vivo* system certainly needs to be addressed. In a recent report, Chandrasekaran and Nagarajan (41) demonstrated that, in rats, plumbagin was excreted within 24 h and was detectable in urine up to 48 h after p.o. administration. In another study, it was shown that the p.o. bioavailability of plumbagin ($AUCp_{.o.}/Dose_{p.o.}$)/($AUC_{i.v.}/Dose_{i.v.}$) was ∼38.7 ± 5% (42). Nevertheless, we hypothesize that as copper is elevated in numerous cancers the amount of bioavailable plumbagin would be sufficient to elicit the cytotoxic response. In view of the results described above and other observations (43,44), we would like to propose that anticancer and apoptosis-inducing property of plumbagin involves its ability to mobilize endogenous copper ions possibly the copper bound to chromatin. Since copper is capable of mediating activation of a variety of phenolic compounds (45), it is reasonable to propose that the DNA-associated copper in cells may have the potential to activate plumbagin via a copper-redox reaction, producing reactive oxygen and electrophilic plumbagin intermediates (semiquinone intermediate). The interaction of plumbagin with DNA-associated copper may finally result in oxidative DNA damage and this might contribute to the cytotoxicity induced by plumbagin.

In conclusion, our data indicate that human skin carcinoma cells are highly sensitive to growth inhibition and apoptosis induction by plumbagin through the generation of ROS via Cu(II)/Cu(I) redox cycle mechanism. More importantly, based on our own observation that the plumbagin does not exhibit any significant toxicity in normal cells as evident from the data shown in our earlier paper and that of many others, we could suggest that plumbagin possesses selectivity between normal and cancer cells. In short, our result strongly suggests that plumbagin is an excellent lead compound in anticancer drug discovery process and these properties of plumbagin could be further explored for the development of anticancer agents with higher therapeutic index, especially for skin cancer.

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Conflict of interest statement: None declared.

References

1. Russo, G. L. (2007) Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochem. Pharmacol.*, **74**, 533–544.
2. Lin, L. C., Yang, L. L. and Chou, C. J. (2003) Cytotoxic naphthoquinones and plumbagic acid glucosides from *Plumbago zeylanica*. *Phytochemistry*, **62**, 619–622.
3. Sandur, S. K., Ichikawa, H., Sethi, G., Ahn, K. S. and Aggarwal, B. B. (2006) Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappa B-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *Biol. Chem.*, **281**, 17023–17033.

4. Wang, C. C. C., Chiang, Y. M., Sung, S. C., Hsu, Y. L., Chang, J. K. and Kuo, P. L. (2008) Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells. *Cancer Lett*., **259**, 82–98.
5. Ahmad, S., Fazal, F., Rahman, A., Hadi, S. M. and Parish, J. H. (1992) Activities of flavanoids for the cleavage of DNA in presence of Cu (II): correlation with the generation of active oxygen species. *Carcinogenesis*, **13**, 605–608.
6. Bhat, R. and Hadi, S. M. (1994) DNA breakage by tannic acid and Cu(II) sequence specificity of the reaction and involvement of active oxygen species. *Mutat. Res*., **313**, 39–48.
7. Ahsan, H. and Hadi, S. M. (1998) Strand scission in DNA induced by curcumin in the presence of Cu(II). *Cancer Lett*., **124**, 23–30.
8. Azmi, A. S., Bhat, S. H. and Hadi, S. M. (2005) Resveratrol-Cu(II) induced DNA breakage in human peripheral lymphocytes: implications for anticancer properties. *FEBS Lett*., **579**, 3131–3135.
9. Linder, M. C. (1991) *Nutritional Biochemistry and Metabolism*. New York: Elsevier Science.
10. Kagawa, T. F., Geiestanger, B. H., Wang, A. H. J. and Ho, P. S. (1991) Covalent modification of guanine bases in double-stranded DNA: 1, 2 Z-DNA structure of d (CGCGCG) in the presence of $CuCl_2$. *J. Biol. Chem*, **266**, 20185–20191.
11. Furst, P. and Hamer, D. (1989) Co-operative activation of a eukaryotic transcription factor interaction between Cu(I) and yeast ACEH protein. *Proc. Natl Acad. Sci. U.S.A*., **86**, 5267–5527.
12. Prutz, W. A., Butler, J. and Land, E. J. (1990) Interaction of Cu(I) with nucleic acids. *Int. J. Radiat. Biol*., **58**, 215–234.
13. Thiele, D. J. (1992) Metal-regulated transcription in eukaryotes. *Nucleic Acids Res*., **20**, 1183–1191.
14. Sagripanti, J. L., Goaring, P. L. and Lamanna, A. (1991) Interaction of copper with DNA and antagonism by other metals. *Toxicol. Appl. Pharmacol*., **110**, 477–485.
15. Dijkstra, M., Kuiper, F., Smit, E. P., De Vries, R., Havinga, R. and Vonk, J. (1991) Biliary secretion of trace elements and minerals in the rat. *J. Hepatol*., **13**, 112–119.
16. Agarwal, K., Sharma, A. and Talukder, G. (1989) Effects of copper on mammalian cell components. *Chem. Biol. Interact*., **69**, 1–16.
17. Swauger, J. E., Dolan, P. M. and Kensler, T. W. (1990) Role of free radicals in tumor promotion and progression by benzoyl peroxide. *Prog. Clin. Biol. Res*., **340**, 143–152.
18. Li, Y. and Trush, M. A. (1993) Oxidation of hydroquinone by copper; chemical mechanism and biological effects. *Arch. Biochem. Biophys*., **30**, 346–355.
19. Driscoll, J. S., Hayard, G. F., Wood, H. B. and Goldin, A. (1974) Structure-antitumor activity relationships among quinone derivatives. *Cancer Chemother. Rep*., **24**, 1–27.
20. Bachur, N. R., Gordon, S. L. and Gee, M. V. (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res*., **38**, 1745–1750.
21. Nazeem, S., Azmi, A. S., Hanif, S. and Kumar, K. S. (2008) Reactive oxygen-dependent DNA damage resulting from the oxidation of plumbagin by a copper-redox cycle mechanism: implications for its anticancer properties. *Austr Asian J. Cancer*, **7**, 65–72.
22. Kumi-Diaka, J., Saddler-Shawnette, S., Aller, A. and Brown, J. (2004) Potential mechanism of phytochemical-induced apoptosis in human prostate adenocarcinoma cells: therapeutic synergy in genistein and beta-lapachone combination treatment. *Cancer Cell Int*., **4**, 5–13.
23. Duke, R. C. and Cohen, J. J. (1992) Morphological and biochemical assays of apoptosis. In Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevack, E. M. and Strober, W. (eds), *Current Protocols in Immunology*. Wiley, New York, pp. 3.17.1–3.17.16.
24. Pool-Zoble, B. L., Guigas, C., Klien, R. G., Neudecker, C. H., Renner, H. W. and Schmezer, P. (1993) Assessment of genotoxic effects by lindane. *Food Chem. Toxicol*., **31**, 271–283.
25. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1998) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*., **175**, 184–191.
26. Tice, R. R., Agurell, E., Andserson, D. *et al*. (2000) Single cell gel/comet assay; guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen*., **35**, 206–221.
27. Melo, A. M., Jardim, M. L., Santana, C. E., Lacet, Y., Filho, J. L., Lima, I. and Leoncio, O. G. (1974) First observations on the topical use of primin, plumbagin and mayteni in patients with skin cancer. *Rev. Inst. Antibiot*., **14**, 9–16.
28. Su, T. T., Chang, H. L., Shyue, S. K. and Hsu, S. L. (2005) Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. *Biochem. Pharmacol*., **70**, 229–241.
29. Antosiewicz, J., Herman-Antosiewicz, A., Marynowski, S. W. and Singh, S. V. (2006) C-Jun NH(2)-terminal kinase signaling axis regulates diallyl trisulfide-induced generation of reactive oxygen species and cell cycle arrest in human prostate cancer cells. *Cancer Res*., **66**, 5379–5386.
30. Pryor, W. A. (1988) Why is hydroxyl radical the only radical that commonly binds to DNA? Hypothesis: it has a rare combination of high electrophilicity, thermochemical reactivity and a mode of production that occurs near DNA. *Free Radic. Biol. Med*., **4**, 219–223.
31. Chevion, M. (1988) Site specific mechanism for free radical induced biological damage. The essential role of redox-active transition metals. *Free Radic. Biol. Med*., **5**, 27–37.
32. Ueda, J., Takai, M., Shimazu, Y. and Ozawa, T. (1998) Reactive oxygen species generated from the reaction of copper (II) complexes with biological reductants cause DNA strand scission. *Arch. Biochem. Biophys*., **357**, 231–239.
33. Rahman, A., Shahabuddin, S., Hadi, S. M., Parish, J. H. and Ainley, K. (1989) Strand scission in DNA induced by quercetin and Cu (II): role of Cu(I) and oxygen free radicals. *Carcinogenesis*, **10**, 1833–1839.
34. Lewis, C. D. and Laemelli, U. K. (1982) Higher order metaphase chromosome structure: evidence for metalloprotiens interactions. *Cell*, **29**, 171–181.
35. Bryan, S. E. (1979) *Metal Ions in Biological Systems*. Marcel Dekker, New York.
36. Ebadi, E. and Swanson, S. (1988) The status of zinc, copper and metallothionen in cancer patients. *Prog. Clin. Biol. Res*., **259**, 167–175.
37. Carpentieri, U., Myers, J. and Thorpe, L. (1986) Copper, zinc and iron in normal and leukemic lymphocytes from children. *Cancer Res*., **46**, 981–984.
38. Yoshida, D., Ikada, Y. and Nakayama, S. (1993) Quantitative analysis of copper, zinc and copper/zinc ratio selective human brain tumors. *J. Neurooncol*., **16**, 109–115.
39. Burkitt, M. J., Milne, L., Nicotera, P. and Orrenius, S. (1996) I,10-Phenanthroline stimulates internuleosomal DNA fragmentation in isolated rat liver nuclei by promoting redox activity of endogenous copper ions. *Biochem. J*., **313**, 163–169.
40. Smets, L. A. (1994) Programmed cell death (apoptosis) and the response to anticancer drugs. *Anticancer Drugs*, **5**, 3–9.
41. Chandresekaran, B. and Nagarajan, B. (1981) Metabolism of echitamine and plumbagin in rats. *J. Biosci*., **4**, 395–400.
42. Yen-Ju, H., Lei-Chwen, L. and Tung-Hu, T. (2006) Measurement and pharmacokinetic study of plumbagin in a conscious freely moving rat using liquid chromatography/tandem mass spectrometry. *J. Chromatogr*., **844**, 1–4.
43. Hadi, S. M., Asad, S. F., Singh, S. F., Singh, S. and Ahmad, A. (2000) A putative mechanism for anticancer and apoptosis inducing properties of plant derived polyphenolic compounds. *IUBMB Life*, **50**, 167–171.
44. Rietgens, I. M. C. M., Boersma, G. M., Haan, L., Spenkelink, B., Awad, M. H. and Cnubben (2002) The pro-oxidant chemistry of the natural antioxidant vitamin C, vitamin E, carotenoids and flavanoids. *Environ. Toxicol. Pharmacol*., **11**, 321–333.
45. Li, Y. and Trush, M. A. (1994) Reactive oxygen dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. *Cancer Res*., **54**, 18955–18985.

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