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

S. Nazeem1,2, Asfar S. Azmi3, Sarmad Hanif4, Aamir Ahmad3, Ramzi M. Mohammad3, S. M. Hadi1 and K. Sateesh Kumar2,*

1Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India, 2Plant Biotechnology Division, Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695562, Kerala, India, 3Department of Pathology and 4Department of Internal Medicine, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201, USA

Plumbagin, a naphthoquinone derived from the medicinal plant Plumbago zeylanica L. has been shown to exert anticancer and anti-proliferative activities in cells in culture as well as in animal tumor models. In our previous paper, we have reported the cytotoxic action of plumbagin in plasmid pBR322 DNA as well as in 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 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 LD50 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 ~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 ~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.

*To whom correspondence should be addressed. Tel: +91 471 237 0907; Fax: +91 471 237 0907; Email: satkrishnan1@yahoo.com

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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 H2O2 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-(Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-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% CO2 in a humidified chamber to prevent drying of the gel and analyzed the kinetics of growth and the experiment was performed essentially according to the method of Singh et al. (22). For SKBR3 cells, lymphocyte DNA breakage was studied after pre-incubating the cells with 20 (5 μl) of MTS–PMS solution followed by incubation at room temperature for 2 h. Upon termination, the supernatant was discarded and pelleted lymphocytes were re-suspended in 10 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 then incubated at 37°C in 5% CO2 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% CO2 incubator for 12–48 h. After 12 h, 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 ELISAPLUS (Roche, Indianapolis, IN) kit using the protocol provided by the manufacturer with minor modifications. SKBR3 cells were plated in duplicate wells at 2.0 × 105 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 with and after trypsinization dead cells were counted by trypsin staining. In independent 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.

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% CO2 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 (>93% viability) were collected as auffy coat after centrifugation 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.

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 CuCl2. 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.
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(1)-specific scavenger (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.
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 CuCl₂ induces extensive DNA breakage in isolated lymphocytes (21). Plumbagin alone at any of the concentrations tested did not damage lymphocyte DNA, whereas on addition 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 CuCl₂. Results of Figure 4 indicate that, with increasing concentration of plumbagin, a progressive increase in DNA breakage [as indicated by increase in tail length (l) mm] 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.

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
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 bond 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$_2$O$_2$ 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, Chandra-sekaran 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./Dosep.o.)/(AUCi.v./Dosei.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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