Plumbagin Induces Cytotoxicity *via* Loss of Mitochondrial Membrane Potential and Caspase Activation in Metastatic Retinoblastoma

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Abstract. *Background/Aim:* We investigated the cytotoxic effects of plumbagin on metastatic retinoblastoma, using the highly metastatic cell line Y79. *Materials and Methods:* Effect of plumbagin on cell growth was assessed with water-soluble tetrazolium 1 (WST-1) cell proliferation assay and automated hemocytometry with trypan blue-exclusion assay. Cell death was studied with acridine orange/ethidium bromide live-dead assay and annexin-V-fluorescein isothiocyanate/propidium iodide microscopy. Loss of mitochondrial membrane potential was studied with JC-10 dye and caspase activation was investigated using CellEvent Caspase-3/7 Green detection reagent. *Results:* Plumbagin highly significantly reduced the growth of Y79 cells treated for 24 h with 2.5 μ M or more. Plumbagin also induced significantly high levels of cell death which was associated with loss of mitochondrial membrane potential and caspase activation. *Conclusion:* At very low concentration (2.5 μ M), plumbagin potently induced cytotoxicity in metastatic retinoblastoma cells via loss of mitochondrial membrane potential and caspase activation.

Although retinoblastoma (RB) is considered a rare neoplasm, it remains the most common primary pediatric intraocular malignancy (1). The etiology of RB is not completely understood. However, the disease originates from the primitive retinal layer (2). Currently, RB has an excellent prognosis, with a 95% 5-year survival rate, however, predominantly only in

developed countries (3) mainly due to improvement in treatment options which include chemotherapy, ophthalmectomy, laser therapy and cryotherapy (4). Despite this, the long-term survivors of RB face a life-long risk of development of secondary cancer, disease relapse and severe cytotoxic effects of chemotherapeutic drugs.

The long-term survival rates of patients with metastatic RB remain unsatisfactory, mainly a result of invasion and metastasis (5). The frequency of metastatic RB ranges from 4.8 to 11% (6, 7) and it remains major contributor to mortality and is associated with life-long poor prognosis (6, 8-11). Frequent common sites of extraocular RB include the orbit, pre-auricular nodes, bones, central nervous system and liver, and each of these may influence the outcome of metastatic RB. For example, RB diagnosis with orbital invasion is associated with a 10- to 27-fold higher risk of metastasis when compared to cases without orbital extension (10-12). The clinical presentations are quite variable and depend on the site or sites involved.

Identifying and testing novel compounds with not only fewer side-effects but also with high therapeutic efficacy is important for improving the prognosis and outcome of patients with metastatic RB and long-term survivors of this malignancy. One such potential agent is plumbagin. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone, plumbagin) is an active compound found in the root of *Plumbago zeylanica* L, an Indian medicinal plant (13). A range of pharmacological activities including antimicrobial, hypolipidemic, and antitumor effects, have been ascribed to this compound (14-16). The antitumor activities have been demonstrated in a wide range of cancer types and cell lines, in *in vitro* and *in vivo* studies (17-21). However, despite the growing body of studies on the antitumor effect of plumbagin, there are no reports on the cytotoxic responses of metastatic retinoblastoma to this compound.

In this study, we investigated the potential cytotoxic effects of plumbagin in metastatic RB, using the highly metastatic RB cell line Y79.

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Key Words: Metastatic retinoblastoma, Y79, plumbagin, cancer.

Figure 1. Effect of plumbagin (PB) on the growth of Y79 cells. A: WST-1 cell proliferation assay showed highly significant decrease in growth of PB-treated cells compared to DMSO controls ($p < 0.0001$). At doses of 2.5 μM PB and above, the growth of Y79 cells remained statistically unchanged ($p > 0.05$). Results are presented as the mean $\pm$ standard error. B: Phase-contrast image showing characteristic cellular morphology of untreated Y79 cells. C: Phase-contrast image depicting compromised membranes of Y79 cells treated with 2.5 μM PB for 24 h. Scale bar = 50 μm .

Methods and Materials

Drug. Plumbagin was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to prepare a 50-mM stock, which was aliquoted and stored at -20°C until used. All working stocks of plumbagin were prepared to a final concentration of 0.01% DMSO.

Cell lines and cell cultures. The human-derived metastatic RB cell line Y79 was obtained from the American Tissue and Cell Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA), 1% L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) and penicillin/streptomycin (Thermo Fisher Scientific). All cells were maintained in a humid environment with 5% CO_2 at 37°C .

WST-1 cell proliferation assay. Cell growth was assessed with WST-1 cell proliferation assay (Roche, Branchburg, NJ, USA), according to the manufacturer's instructions and as described by Gharbaran *et al.* (22). One hundred microliters of medium containing cells at 1×10^5 cells/ml were seeded in a 96-well plate, and incubated overnight. Cells were then treated with 0, 1.25, 2.5, 5, 10 and 20 μM of plumbagin or DMSO for 24 h. The treated cells were incubated with 10 μl WST-1 reagent for 3 h following standard cell culture conditions. Absorbance was read at 450 nm on a Synergy H1 Hybrid microplate reader (BioTek Instruments, Winooski, VT, USA). Cell growth (as a percentage) was computed as a ratio of the absorbance (A450) of treated (either plumbagin or DMSO) cells to the absorbance of the untreated (0 μM) control. The assay principle is based on the conversion of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes.

Acridine orange (AO) and ethidium bromide (EtBr) live/dead assay. AO/EtBr assay was used to determine cell death. Five hundred microliters of medium containing cells at 2×10^5 cells/ml were seeded in poly-L-lysine (Sigma-Aldrich)-coated 20-mm microwells of 35 mm petri dish (MatTek Corporation, Ashland, MA, USA) overnight and then treated with 0 and 2.5 μM plumbagin, for 24 h. Twenty microliters of a solution consisting of 100 $\mu\text{g/ml}$ each of AO and EtBr were then added to each well and dishes were

immediately imaged. In this assay, the membrane-permeable AO-stained live cells green and EtBr, which is membrane-impermeable, stained the nuclei of dead cells orange to red.

Annexin V fluorescein isothiocyanate (FITC)-propidium iodide (PI) assay. Annexin V-PI staining was carried out using Annexin V FITC Assay Kit (Cayman Chemicals), according to the manufacturer's instructions. One hundred microliters of medium containing cells at 1×10^5 cells/ml were seeded in 96-well plates overnight and treated with 0 and 2.5 μM plumbagin for 24 h. Treated cells were transferred to a microfuge tube and collected by centrifugation at $400 \times g$ for 5 min at room temperature, and then resuspended in 100 μl 1 X binding buffer. Cells were then centrifuged as described and incubated in 50 μl annexin V FITC/PI staining solution for 10 min at room temperature in a dark chamber. Stained cells were collected by centrifugation and resuspended in binding buffer. Cells were then transferred to chamber slides containing poly D-lysine for analysis by microscopy.

Detection of mitochondrial membrane potential (Ψ_m). Changes in Ψ_m were assessed by staining treated cells with JC-10 dye according to the manufacturer's (Sigma-Aldrich) instruction. Five hundred microliters of medium containing cells at 1×10^5 cells/ml was seeded and treated as described for AO/EtBr assay. The cells were then incubated in JC-10 Dye Loading Solution for 30 min following standard cell culture condition. An aliquot (250 μl) of the JC-10 Dye Loading Solution-medium mix was withdrawn and replaced with 250 μl Assay Buffer B. Cells were immediately imaged.

Detection of caspase activities. Caspase 3/7 activities were analyzed using CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies, CA, USA), according to manufacturer's instructions. Cells were seeded and treated as described for AO/EtBr live-dead assay. Cells were then incubated with CellEvent Caspase-3/7 Green Detection Reagent at a final concentration of 4 μM for 30 min, following standard cell culture conditions.

Microscopy. Microscopy was carried out as previously described (22). Images for live/dead assay, caspase 3/7 activation, and annexin V-FITC/PI analyses were generated from randomly selected fields. Approximately 5 to 15 fields were imaged per dose. Cell counts on

Figure 2. Detection of plumbagin (PB)-induced cell death with acridine orange/ethidium bromide (AO/EtBr) live-dead assay. A: AO/EtBr staining of Y79 cells treated with PB at 0 and 2.5 μ M for 24 h. B: AO/EtBr staining revealed a significantly higher proportion of dead cells (EtBr+, red) due to PB treatment compared to untreated cells (0 μ M) ($p < 0.0001$). Results are presented as the mean $\pm$ standard error. Scale bar = 50 μ m.

images were carried out manually, blindly, by independent counters. However, for AO/EtBr and caspase detection assays, the cell count was carried out on the upper left quadrant of each field.

Statistical analyses. Data analyses were performed using StatView 5 (Cary, NC, USA). Analysis of variance was used to determine significant differences between the means as defined by $p < 0.05$. Data obtained from the assays on cell growth, AO/EtBr, annexin V-PI, caspase 3/7, are presented as the mean and standard error of the mean. For the cell growth assay, the mean value was determined from triplicates per dose. Each experiment was repeated at least three times.

Results

Effect of plumbagin on cell growth of metastatic retinoblastoma. As a first step, we tested different concentrations of plumbagin on the growth of Y79 cells treated for 24 h. WST-1 cell

proliferation assay showed plumbagin potently and significantly inhibited cell growth at doses of 2.5 μ M and above compared to cells treated with DMSO (vehicle) equivalent (Figure 1A). However, the plumbagin-induced inhibition of cell growth was not dose-dependent; there was no statistical difference in cell growth for doses of 2.5, 5, 10, and 20 μ M. We also assessed cell growth independently with automated hemocytometry *via* trypan blue exclusion assay. The results were similar to those of the WST-1 cell proliferation assay (data not shown). Phase-contrast microscopy showed plumbagin-treated cells with compromised membranes which was absent from untreated and DMSO controls (Figure 1B and C, respectively), suggesting loss of cell viability associated this compound. In these experiments, the lack of effect on cell growth by the DMSO equivalent indicates that the solvent did not affect cell viability nor did it cause cytotoxicity to the Y79 cells. As a result, we

Figure 3. Detection of apoptotic cells with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. A: Detection of apoptotic Y79 cells after treatment with plumbagin (PB) at 0 and 2.5 μ M for 24 h by annexin V-FITC/PI staining. B: A significantly higher proportion of annexin V-FITC/PI+ cells (stained green and red, respectively) was detected for Y79 cells treated with 2.5 μ M PB compared to untreated cells. Results are presented as the mean $\pm$ standard error. Scale bar in A=50 μ m.

focused our attention on cytotoxicity induced by plumbagin at the lower dose (2.5 μ M) in subsequent experiments.

Plumbagin induced cell death. We next determined whether plumbagin-induced reduced cell growth was partly a consequence of cell death. AO-EtBr staining showed significant cell death (red-orange) in cells treated with 2.5 μ M of plumbagin for 24 h (Figure 2A). As shown in Figure 2B, the mean proportion of EtBr+ cells on 24-h treatment with 2.5 μ M plumbagin was 43.50% ($\pm$ 2.19%) compared to the untreated control (7.69% $\pm$ 3.31%) (p <0.0001). plumbagin-induced cell

death was further investigated with Annexin V-FITC/PI microscopy. As shown in Figure 3, Annexin V-PI positive cells were significantly higher in plumbagin-treated Y79 cells (62.97% $\pm$ 2.80) compared to 0 μ M (4.86% $\pm$ 0.93%) (p <0.0001).

Plumbagin reduced Ψ_m . Loss of Ψ_m is an early biochemical indicator of apoptotic cell death. To determine the effects of plumbagin on the function of mitochondria, $\Delta\Psi_m$ was assessed after 24 h treatment of Y79 cells. JC-10, a cationic dye which accumulates potential-dependently in mitochondria, was used to study changes in Ψ_m . Under the microscope, JC-

Figure 4. Plumbagin (PB) induces loss of mitochondrial membrane potential. Staining of Y79 cells treated with 2.5 μ M PB for 24 h with JC-10 dye showed loss of mitochondrial membrane potential (absence of red fluorescence and presence of green fluorescence – lower panel) compared to untreated (0 μ M) cells (presence of both red and green fluorescent signals in upper panel). Scale bar=50 μ m.

JC-10 fluoresces green (540 nm) when the Ψ_m is lower than 140 mV and at higher membrane potential, the dye fluoresces red. With this dye, the loss of Ψ_m is followed by a red-to-green shift. Therefore, microscopically, live cells stained with JC-10 dye contain red puncta or aggregates which are absent from or diminished in dead cells, which stain predominantly green with the dye. In our experiments, control cells (0 μ M plumbagin) exhibited red aggregates which were absent from cells treated plumbagin at 2.5 μ M for 24 h (Figure 4).

Caspase activation. Activation of the caspase cascade is another important molecular hallmark of apoptosis. To determine whether plumbagin-induced cell death progresses *via* caspase activation, we stained Y79 cells incubated with plumbagin for 24 h with a caspase 3/7-specific fluorochrome detection dye. The staining revealed a significantly higher proportion of caspase 3/7-positive (stained green) plumbagin-treated Y79 cells (50.71% $\pm$ 4.93%) compared to untreated controls (5.11% $\pm$ 0.63%) (p <0.0001) (Figure 5).

Discussion

Although RB has an excellent prognosis, with a 95% 5-year survival rate, this is predominantly only for patients in developed countries (3), the prognosis in developing countries remaining relatively poor. In addition, long-term survivors of RB with metastatic phenotype bear the risk of

emergence of secondary neoplasms, and life-threatening toxicities directly related to chemotherapy. To address these concerns, one line of RB research involves the identification of novel agents with less toxicity but high efficacy.

In the current study, we demonstrated that plumbagin potently induced cytotoxicity in metastatic RB and this process was associated with loss of mitochondrial membrane potential and caspase activation. Our WST-1 cell proliferation assay showed that plumbagin significantly restricted the growth of Y79 cells at 2.5 μ M, after 24 h, an indication of the strong antitumor potency of this compound. Cell growth at higher doses of plumbagin was not statistically different from that at 2.5 μ M. The plumbagin-induced growth inhibition noted in our study differs from that of some other reports. *In vitro* study showed that plumbagin inhibited growth of esophageal cancer cells in a dose-dependent manner (at comparable doses as our study) (23). Studies in other cancer types also report dose-dependent inhibition of cancer cell growth by plumbagin (24, 25).

Induction of apoptosis is an important hallmark in cancer therapy. In multiple cancer types, plumbagin limits cell growth by inducing apoptosis (26-30). In our study, treatment of Y79 metastatic RB cells with plumbagin for 24 h resulted in statistically significant levels of cell death, as evident by our AO-EtBr live-dead staining and annexin V assays. This level of cell death was evident at 2.5 μ M plumbagin and above.

Figure 5. Effect of plumbagin (PB) on caspase 3/7 (Casp3/7+) activity in Y79 cells. A: Detection of Casp3/7+ -positive (green) cells after treatment for 24 h with 0 and 2.5 μM PB. B: A highly significant proportion of Casp3/7+ cells were detected in PB-treated cells compared to untreated cells ($p < 0.0001$). Results are presented as the mean $\pm$ standard error. Scale bar = 50 μm .

In general, programmed cell death is driven by two types of apoptotic pathways, namely the death receptor-dependent extrinsic pathway and a mitochondrial-dependent intrinsic pathway (31). Mitochondrial-mediated apoptosis involves disruption of mitochondrial membrane, resulting in loss of Ψm , and cytochrome c release from mitochondria into the cytosol (32). One consequence of this is the activation of the caspase 9/caspase 3 pathway, leading to chromatin condensation and DNA fragmentation (33). In our study, loss of Ψm was observed after plumbagin treatment, as evident by either the reduction or absence of red fluorescence in treated cells compared with untreated cells, following staining with JC-10 dye. In addition, in our study, plumbagin-treated cells had statistically significantly higher levels of caspase-

positive cells (Figure 5) following staining with a caspase 3/7-specific dye. These results suggested that plumbagin-induced apoptosis in metastatic RB likely involves the loss of Ψm and caspase 3/7 activation, which are associated with the mitochondrial-mediated intrinsic cell death pathway.

There are some limitations of the current study. The lack of cell-cycle analyses precludes determination of whether plumbagin induces cell-cycle arrest, and by extension, studies into the mechanism(s) of action. In addition, assaying for cytochrome c release, which is associated with loss of Ψm , was not carried out. Moreover, other hallmarks of apoptosis such as chromatin condensation and DNA fragmentation were not studied. Despite these limitations, the results from our cytotoxic assays highlight the significance

of plumbagin as a potential agent that can be developed for treating metastatic RB. However, future studies are required to determine the molecular details of plumbagin-induced cell death and potential cell-cycle arrest in this malignancy.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

RG conceptualized the project, designed and conducted experiments, interpreted data and wrote the article. OO and CS conducted experiments. RG, OO and SR interpreted data. All Authors read and approved the final article.

Acknowledgements

Support for this project was provided in part by a PSC-CUNY Award, jointly funded by The Professional Staff Congress and The City University of New York (Award #: TRADB-48-360), and The City University of New York Community College Research Grant (Award #: 80212-03-17).

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Received July 17, 2021

Revised August 9, 2021

Accepted August 26, 2021