D-Pinitol treatment induced the apoptosis in human leukemia MOLT-4 cells by improved apoptotic signaling pathway

Xiangmei Yao\textsuperscript{a}, Keqian Shi\textsuperscript{a}, Yanmei Yang\textsuperscript{a}, Xuezhong Gu\textsuperscript{a}, Weiwei Tan\textsuperscript{b}, Qi Wang\textsuperscript{a}, Xiaoli Gao\textsuperscript{a}, Vishnu Priya Veeraraghavan\textsuperscript{c}, Surapaneni Krishna Mohan\textsuperscript{d}, Song Jin\textsuperscript{e,*}

\textsuperscript{a}Department of Hematology, The First People’s Hospital of Yunnan Province, Kunming, Yunnan 650032, China
\textsuperscript{b}Department of Hematology, The People’s Hospital of Chuxiong Yi Autonomous Prefecture, Chuxiong, Yunnan 675000, China
\textsuperscript{c}Department of Biochemistry, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai 600 077, India
\textsuperscript{d}Department of Biochemistry, Panimalar Medical College Hospital & Research Institute, Varadharajapuram, Poonamallee, Chennai 600 123, India
\textsuperscript{e}Department of Rheumatism Immunology, The First People’s Hospital of Yunnan Province, Kunming, Yunnan 650032, China

Abstract

Cancer is still remain as a global burden with the 18.1 million and 9.6 million new cases and mortalities, respectively estimated globally. Leukemia may arise at all ages varied from the infants to elders. In this exploration, we planned to evaluate the antiproliferative effect of D-pinitol on human leukemia MOLT-4 cells. Anticancer potential of D-pinitol was examined using MTT assay. Reactive oxygen species (ROS) generation was studied by fluorescence microscopic method using DCFH-DA staining. Apoptotic morphological alterations were determined by dual staining (acridine orange and ethidium bromide). Western blot and ELISA methods were employed to study apoptotic protein expression. D-pinitol treatment significantly induced cytotoxicity in human leukemia MOLT-4 cells. We observed that D-pinitol induces the generation of ROS in MOLT-4 cells. Further, we noticed that D-pinitol significantly induced apoptosis in a dosage dependent manner. Moreover, western blot and ELISA based analysis revealed that D-pinitol elevated the Bax, Caspase-3, Caspase-9 and attenuated the Bcl-2 expression in leukemic cancer cell. Our findings suggest that D-pinitol treatment induces the apoptosis in human leukemic cells by generating intracellular ROS and modulating apoptotic protein expression.

1. Introduction

Cancer is distinguished by the abnormal growth and multiplication of cells and remains a global burden. According to statistics from GLOBOCAN, 2018, there were about 18.1 million and 9.6 million new cases and mortalities, respectively estimated globally. Among them, 437,033 number of new leukemia cases and 309,006 numbers of leukemia death was recorded (Bray et al., 2018). Several malignant disorders with a characteristic of increased numbers of leucocytes in blood and/or bone marrow collectively called as leukemia. Leukemias may arise at all ages varied from the infants to elders. Acute lymphoblastic leukemia is common in infancy; acute myeloid leukemia is frequent in older peoples; chronic myeloid leukemia is infrequent in young children; chronic lymphocytic leukemia is general form of leukemia in Western world (Juliussen et al., 2016).

Childhood leukemia is the most common diagnosed among all other leukemia worldwide. The excessive level of radiation and contact to the environmental radiation are identified as a influenced risk factor for leukemia (Belson et al., 2006). Whereas, DNA damaging therapy and previously diagnosed hematologic malignancy is more prone to develop AML. Therapy-linked ailments is primarily due to the chemotherapy, like topoisomerase II-inhibitors, alkylating agents and radiation, which is employed to treat the breast cancer and lymphoma, and is thus more frequent in elder peoples (Sill et al., 2011). Topoisomerase II-inhibitors and alkylating agents like chemotherapeutic agent ends up with therapy related disease. About 8% of adult AML patient has been reported for therapy related disease (Sasaki et al., 2016). Apart from these some of genetic factors such as...
Down's syndrome, germline mutations and ETV6 have also been documented to be linked with an augmented risk.

The intrinsic apoptotic pathway is activated in reaction to cellular stimuli, including failure in a repair of DNA damage, hypoxia, oxidative stress, high intracellular calcium (Elmore et al., 2007). Mitochondria play a crucial function in orchestrating intrinsic apoptosis, by regulating the stability between pro-apoptotic and anti-apoptotic family of proteins. When the cells receive intracellular apoptotic signals, the pro-apoptotic overwhelm the anti-apoptotic proteins, resulting in augmented permeability of the mitochondrial membrane that finally results the cytochrome-c release into the cytoplasm. Later, it forms the multi-protein complex called as apoptosome. This apoptosome complex cleaves the pro-caspases into its active caspases, which are responsible for the morphological and biochemical changes during the apoptotic process (Wang et al., 2009). The extrinsic death receptor pathway is stimulated in response to extracellular stimuli-binding of death ligands such as TRAIL (TNF-related apoptosis inducing ligand), FasL, TNF (Tumor Necrosis Factor). Once the death ligands binds with their corresponding receptors, it forms DISC (Death Inducing Signaling Complex). This DISC has an ability to induce apoptosis through activating caspases-8 (Giucciardi et al., 2009).

Scientific and research interest in identification of novel agents from naturally derived drugs because of their less toxic side effects and health promoting activities (Cragg et al., 2013). Plants have been a prehistoric narrations of their usage in folk medicines. The flavonoids are secondary metabolite most commonly found in plants. It is well known for its versatile health benefit for example antioxidant activity, anti viral and anti bacterial infection, antiinflammatory activity (Kumar et al., 2013). The vegetables and fruits containing rich flavonoids consumption are reported to prevent various types of human cancer in experimental models. D-pinitol is an active component of soybean. In soybean meal, it is stimulated in response to extracellular stimuli-binding of death ligands such as TRAIL (TNF-related apoptosis inducing ligand), FasL, TNF (Tumor Necrosis Factor). Once the death ligands binds with their corresponding receptors, it forms DISC (Death Inducing Signaling Complex). This DISC has an ability to induce apoptosis through activating caspases-8 (Giucciardi et al., 2009).

2. Materials and methods

2.1. Chemicals

D-pinitol, dulbecco’s modified eagle medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other chemicals, fluorescent stains, and reagents were procured in Sigma-Aldrich, USA. The kit and antibodies for western blotting were procured from the Biorad, Germany and R&D Biosystem, China, respectively.

2.2. Cell culture and condition

The anticancer potential of D-pinitol was inspected against the human leukemia (MOLT-4) cells as described previously (Lu et al., 2018). In brief, from the parental flask, cells were collected by trypsinization and 1 × 10^6 cells per well were loaded in 6 well plates. Then, cells were supplemented with 25 and 50 μM of D-pinitol then maintained for 24 h. After that cells were thoroughly washed thrice with PBS at 5 min interval. Then, 5 μl of DCFH-DA (1 mg/ml) was mixed and incubated under dark for 30 min at 37 °C. Excessive dyes were removed by repeated washing with PBS. Atlast, cells were examined with the aid of the fluorescence microscope and images were photographed.

2.3. Cell viability assay

The cytotoxic level of D-pinitol against the human leukemia MOLT-4 cells was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) based colorimetric assay (Liu et al., 1997). Cells were harvested from the exponentially grown stock culture and 10,000 numbers of cells per well loaded in 96 well plate. The cells were allowed for 24 h incubation at 5% CO2 chamber at 37 °C. After 24 h of incubation, cells undergone with various concentration of D-pinitol (from 5 to 100 μM) treatment and incubated for additional 24 h. After the treatment procedure, 100 μl of MTT solution was amalgamated and the incubation was continued for further 4 h. Finally, the DMSO (100 μl) was mixed to dissolve the MTT crystal and to stop the reaction. The absorbance were made at 570 nm.

2.4. Detection of intracellular ROS

Qualitative analysis of intracellular ROS were determined by using a fluorescent probe named 2, 7-dicacetylchlorofluorescein (DCFH-DA) in control and D-pinitol treated human leukemia (MOLT-4) cells as described previously (Lu et al., 2018). In brief, from the parental flask, cells were collected by trypsinization and 1 × 10^6 cells per well were loaded in 6 well plates. Then, cells were supplemented with 25 and 50 μM of D-pinitol then maintained for 24 h. After that cells were thoroughly washed thrice with PBS at 5 min interval. Then, 5 μl of DCFH-DA (1 mg/ml) was mixed and incubated under dark for 30 min at 37 °C. Excessive dyes were removed by repeated washing with PBS. Finally, cells were inspected under the fluorescence microscope and images were taken.

2.5. Detection of apoptotic morphological changes by acridine orange and etidium bromide (AO/EtBr) staining

D-pinitol stimulated apoptotic morphological alterations were studied in human leukemia MOLT-4 cells by employing AO/EtBr dual staining followed by the earlier methods Aithal et al., (2009). Briefly, from the parental flask, cells were collected by trypsinization and 1 × 10^6 cells per well were loaded in 6 well plates. Then, cells were supplemented with D-pinitol for 24 h. After that cells were thoroughly washed thrice with PBS at 5 min interval. Then, 1 μl of AO/EtBr (5 mg/ml concentration) was amalgamated and incubated under dark for 30 min at 37 °C. Excessive dyes were removed by repeated washing with PBS. Finally, cells were inspected under the fluorescence microscope and images were taken.

2.6. Western blot analysis

Proteins from the untreated normal and D-pinitol supplemented human leukemia MOLT-4 cells were separated with the aid of RIPA buffer, which consists of protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA) in accordance with the instructions of manufacturer. The samples containing 50 μg of protein were boiled for 5 min with loading buffer and size-separated through 10% SDS-PAGE gel. Then gel was blotted onto a nitrocellulose membrane via the semi-dry equipment (Biorad, Germany). The membrane was incubated with 5% BSA at 4 °C for overnight. Later than washed with TBST and relevant primary monoclonal antibodies for Bcl-2, Bax, Cas-9, and Cas-3 were added (dilution 1:1000) and sustained for 5–6 h at 37 °C. Then secondary antibodies coupled with horseradish peroxidase (dilution 1:2000) was added for 2 h at 37 °C. Lastly, enhanced chemiluminescence method were employed to detect the bands (Biorad, Germany). The images were attained through Image Studio software (LI-COR) (Dalmau et al., 1990).
2.7. ELISA assay for apoptosis

The expression status of apoptotic proteins were examined through the respective ELISA test kits (R&D Biosystem, China) as mentioned previously by Chen et al. (2014).

2.8. Statistical analysis

Statistical assessments were executed in the SPSS (ver.16) statistical tool. Data were illustrated as mean ± standard deviation (SD). The one way analysis of variance (ANOVA) subsequently Duncan Multiple Range Test (DMRT) comparison test was executed to compare the statistical variations between the test groups. Data was regarded as significant if p value are less than 0.05.

3. Result and discussion

Cancer in humans has been recognized for age back in prehistoric periods (David et al., 2010). Currently, it accounts for about 18.1 million new incidences and 9.6 million mortalities estimated worldwide (Bray et al., 2018). Defeating cancer is being continuously failed, because cancer is a complex disease arise from different factors including environmental and genetic. However, still the treatment for is largely based on chemotherapeutic drugs to eliminate, reduce and also to alleviate pain (Tian et al., 2018). Chemotherapeutic agents are targeted to induce cell death in cancer cells by inducing DNA damage. The major limitation of these anticancer agents is often leads to disease recurrence (Sawicka et al., 2016). The next and most important limitation is an adverse toxicity of non-targeted organs or tissues.

Most of the clinical anticancer drugs work by damaging DNA. Such a DNA damaging chemotherapeutics like alkylating agent topoiso merase II-inhibitors, platinum derived drugs and radiation therapy or combination of these modalities are considered as complication and risk factors for different types of leukemia. Therapy-linked acute myeloid leukemia and myelodysplastic syndrome are well recognized clinical syndrome turn out into a late hurdle following the cytotoxic treatments (Goldstein et al., 2015). The distinctiveness of therapy induced leukemia primarily depends on the particular agents in addition to the collective dosage and strength of the radiotherapy. With a view to limiting these adverse effects of existing anticancer agent like cisplatin, new platinum derived anticancer drugs were developed (Hambley, 1997). Though they were successful in terms stability, unfortunately requires a higher dosage compared to cisplatin, ultimately ends up with myelosuppression (Kelland et al., 2007). The same kind of observation can also be seen in radiotherapy which stimulates rapid repopulation in cancer in vitro and in vivo models.

Over the four decades of time, there has been the continuous search for the small molecules from naturally occurring sources like plant or microbes chemotherapeutic drugs. Natural products displayed an significant benefits in establishing the cancer chemotherapeutic agents, either as its original/unmodified or synthetically modified forms. More than 50% FDA approved anticancer agents derived from natural products (Clark et al., 1996). In this current exploration, we inspected the anticancer activity of D-pinitol, a naturally occurring flavonoid against human leukemic cancer MOLT-4 cells. The level of the cytotoxicity of D-pinitol against the MOLT-4 cells investigated by the MTT assay (Fig. 1). D-pinitol at various concentrations (5–100 μM) for 24 hr treatment showed gradual decrease in cell proliferation. Further, we observed that D-pinitol inhibited over 50% of the cell growth at 25 μM dosage for 24 h. Further, we evaluated the ROS inducing ability of D-pinitol using fluorescent microscopic methods (Fig. 2). We noticed that D-pinitol effectively induces ROS generation in human leukemia MOLT-4 cells in a dosage reliant mode. It is well docu-

![Fig. 1. Effect of D-Pinitol induced cytotoxicity on human leukemic MOLT-4 cells. Cells (1X10⁵ cells/well) were treated with increasing concentrations of D-Pinitol ranging from 5 to 100 μM for 24 h and then assessed for cell viability using MTT assay. Values are given as means ± S.D. of three experiments in each group. significantly at *P ≤ 0.05(DMRT).](image1)

![Fig. 2. Effect of D-Pinitol on intracellular ROS generation using DCFH-DA staining. Photomicrograph of D-Pinitol treated MOLT-4 cells show enhanced green fluorescence under a green lamp (20×).](image2)
mented that flavonoids undergo autoxidation thereby produces superoxide anions. Moreover, peroxidases can metabolize the phenol ring resulting in production of phenoxyl radicals (Nimse et al., 2015). Hence, we conclude that this might be the reason for D-pinitol mediated ROS and cytotoxicity in human leukemia MOLT-4. We examined the apoptosis inducing effect of D-pinitol on human leukemia MOLT-4 cells using AO/EtBr staining. The AO/EtBr staining results showed that D-pinitol treatment significantly induces the apoptosis in human leukemia MOLT-4 cells (Fig. 3). Further, we revealed that the D-pinitol mediated apoptotic effect was concentration dependent manner. The fluorescent microscopic images clearly shows that there were no apoptotic changes in untreated control cell (Fig. 3).

The Bcl-2 family of proteins performs a critical function in regulating the intrinsic mitochondrial apoptotic pathway. The modulation of Bcl-2 family proteins is frequently reported in human cancer. Recent progress made in understanding Bcl-2 family proteins or perturb mitochondrial integrity leads to development novel anticancer therapies that target Bcl-2. Many anticancer agents including phytochemicals mostly triggers the mitochondria mediated cell death through inhibiting Bcl-2 and activating Bax (Guo et al., 2003). In our finding, we noticed that D-pinitol increased the Bax and diminished the Bcl-2 activity in a dose reliant mode (Fig. 4). Our outcomes were coincident with previous findings reported (Rengarajan et al., 2014). Hence, we conclude that increasing free radical generation and direct activation Bax might be responsible for this reported activity. In this investigation, further, we inspected the potential of D-pinitol induced apoptosis associated caspase mediated responses in MOLT-4 cells. Because, the caspase-3 is known to execute the apoptotic process. Results from the western blot analysis revealed that D-pinitol

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**Fig. 3.** Effect of D-Pinitol induced apoptosis using AO/EtBr staining. Photomicrograph of D-Pinitol treated MOLT-4 cells showed typical morphological changes and fragmented DNA. Images were taken under fluorescence microscope (20×).

**Fig. 4.** D-Pinitol induced apoptosis in MOLT-4 cells. Cells treated with D-Pinitol for 24 h with or without were collected. Immunoblots were performed with cell lysate to determine the apoptotic protein expression such as Bax, Bcl-2, caspase-9 and caspase-3 by western blot analysis.

**Fig. 5.** D-Pinitol induced Caspase-3 & 9 expression using ELISA method in human leukemia MOLT-4 cells. Values are depicted as mean ± S.D. of three individual experiments in each group. Statistical significance at *P* ≤ 0.05(DMRT).
effectively induced caspases – 3 & 9 in a dosage dependent mode in MOLT-4 cells (Fig. 5). Similarly, it is important to observe that D-pinitol is already reported for inducing caspases and proapoptotic proteins like Bax in a concentration dependent fashion in breast cancer cells. This was further confirmed by fluorescent based apoptotic staining studies. In conclusion, our studies suggest that D-pinitol induces apoptosis via modulating proapoptotic protein expression through generating ROS in human leukemia MOLT-4 cells. However the further studies were still needed in the future to understand the exact therapeutic mechanisms of the D-pinitol against the leukemia.

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