**Vernodalol mediates antitumor effects in acute promyelocytic leukemia cells**

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**Abstract.** Acute promyelocytic leukemia (APL) remains a challenge to cure due to the side effects of cytotoxic chemotherapy and drug resistance. The present study demonstrated that vernodalol, an active compound isolated from *Centratherum anthelminticum*, suppresses APL cell proliferation and induces cell cycle arrest in the G2/M phase through the upregulation of p21 and cell division cycle 25. In addition, vernodalol induced cellular apoptosis via the mitochondrial pathway as observed by the cleavage of caspase-9 as well as the release of cytochrome c and Smac/DIABLO into the cytosol. A mechanistic study revealed that vernodalol may exert its antitumor activity through the suppression of phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin signaling. In conclusion, vernodalol may be developed as a potential therapeutic compound for the treatment of APL.

**Introduction**

Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemia (AML) that results from an arrest of the terminal differentiation of promyelocytes into granulocytes (1,2). APL accounts for 5-15% of all cases of AML and is frequently associated with the expression of the oncogenic promyelocytic leukemia-retinoic acid receptor α fusion gene (3,4). Clinically, APL is now considered a fatal disease with a high rate of early mortality, often due to hemorrhage from disseminated intravascular coagulation (DIC) or hyperfibrinolysis (5). Chemotherapy with anthracyclines, including daunorubicin, idarubicin and cytarabine arabinoside, which are the frontline treatments of APL, resulted in complete remission in 75-80% of patients (6,7). However, this standard treatment regimen was associated with a high early mortality rate due to the exacerbation of pre-existing DIC (5,7,8). Despite high sensitivity to anthracyclines, only 35-45% of patients with APL are cured by standard chemotherapy alone (9). Therefore, it is urgently required to develop novel therapeutic agents for patients with APL to overcome the high early mortality rate.

*Centratherum anthelminticum,* commonly termed kalajiri, somraj, black cumin or bitter cumin, is a robust leafy plant belonging to the Asteraceae family of flowering plants (10). The plant has additional scientific synonyms, including *Vernonia anthelmintica* and *Conyza anthelmintica.* It is widely used as an herbal medicine against cough, fever and diarrhea in a number of regions, including India, the Himalaya mountains, Khasi Hills, Sri Lanka and Afghanistan (11). The extracts from the seeds of *C. anthelminticum* have been demonstrated to possess various pharmacological properties, including analgesic, antipyretic, anti-inflammatory, antiviral and anti-filarial effects (12-15). A previous study reported that the chloroform fraction, but not the hexane or methanol fractions, from *C. anthelminticum* seeds exhibited antioxidant properties of inhibiting tumor necrosis factor-α-induced human cancer cell growth by interrupting the activation of nuclear factor-κB activation (16). Vernodalin is an active compound isolated through bioassay-guided fractionation, which can induce apoptosis in human breast cancer cells via the caspase pathway (10). In addition, vernodalin can induce cell cycle arrest and apoptosis in breast cancer cells through forkhead box O3a (11). Vernodalol is similar to vernodalin, and both can be extracted from *C. anthelminticum* (10). However, the effects of vernodalol on carcinoma cells have not been studied.

The present study investigated the antitumor effects of vernodalol on APL cells. The effect of vernodalol on the APL cell cycle and apoptosis was assessed, and the associated molecular mechanisms were investigated. Results of the present study indicated that vernodalol may be utilized as a potent medicine for the treatment of APL.
Materials and methods

Reagents and cell culture. Vernodalol was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies specific for caspase-9 (cat. no. CST 9502), caspase-3 (cat. no. CST 9662), cleaved-poly (ADP ribose) polymerase (PARP; cat. no. CST 9542), B-cell lymphoma-2 (Bcl-2; cat. no. CST 2872), Bcl-2-associated X protein (Bax; cat. no. CST 2774), Bcl-2-associated death promoter (Bad; cat. no. CST 9292) and Bcl-2 homologous antagonist killer (Bak; cat. no. CST 3814) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies specific for phosphatase and tensin homolog (PTEN; sc-7974), myeloid cell leukemia-1 (Mcl-1; sc-12756), Bcl-2-like protein 1 (Bim; sc-130511), cytochrome c (sc-13561) and second mitochondria-derived activator of caspase/direct IAP-binding protein with low pl (Smac/DIABLO; sc-22766) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies specific for phosphorylated (p)-protein kinase B (Akt; ab38449), Akt (ab8805), phosphoinositide 3-kinase (PI3K; ab86714) and mechanistic target of rapamycin (mTOR; ab2732) were purchased from Abcam (Cambridge, MA, USA). Anti-GAPDH antibody was obtained from Sigma-Aldrich (Merck KGaA; G9545). The Annexin V/propidium iodide (PI) binding kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals of analytical grade were obtained from Sigma-Aldrich (Merck KGaA). Human APL cell lines, KG-1a, NB-4 and HL-60, were obtained from Shanghai Centre of Cell Resource, Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained at 37°C in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO₂. All cells were passaged every 3 days.

Cell viability assay. The effects of vernodalol on viability of human APL cells were analyzed by MTT assay (Sigma-Aldrich; Merck KGaA). Briefly, 5x10³ cells per well were plated on 96-well plates and treated with vernodalol at various concentrations (20, 40, 60, 80 and 100 μM) for the indicated times (24, 48 or 72 h). The RPMI-1640 with the compounds or 0.5% dimethyl sulfoxide (DMSO; as the control treatment) was then replaced with 180 µl of fresh media with 10% non-fat milk at room temperature and washed twice with cold 1X PBS. The cell suspension was centrifuged at 377 x g for 15 min at 4°C. The supernatant was removed and stored at -20°C as the cytosolic fraction.

Western blot analysis. The cells were treated with vernodalol at different concentrations (0, 50, 75 or 100 μM) for the indicated time (24 h). The cells were then lysed using Cell Lysis and Mitochondria Intact buffer (250 mM sucrose, 80 mM KCl and 50 μg/ml digitonin in PBS) on ice for 5 min. The cell suspension was centrifuged at 377 x g for 5 min at 4°C. The total protein concentration was determined with bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA) for removal of RNA in a 37°C water bath for 15-20 min. The cells were then analyzed by flow cytometry (FACS Calibur; BD Biosciences). The results are presented as mean values from three independent experiments.

Cell apoptosis analysis. The cells were cultured (5x10⁵) in each well of 6-well plates to 70-80% confluence with RPMI-1640 and then treated with vernodalol at various concentrations (0, 50, 75 or 100 μM) for the indicated time (24 h). The vernodalol-treated and control cells (treated with DMSO only) were harvested by centrifugation for 5 min at 377 x g and room temperature and washed twice with cold 1X PBS. The cells were then stained with 5 μl Annexin V and PI (BD Biosciences) for 15 min in dark conditions at room temperature according to the manufacturer's protocol and then subjected to analysis by flow cytometry (FACS Calibur; BD Biosciences). Data were analyzed using Flowjo software version 10.0 (Tree Star, Inc., Ashland, OR, USA). Early apoptosis was evaluated based on the percentage of cells with Annexin V+PI staining, and late apoptosis was evaluated based on the percentage of cells with Annexin V+/PI+ staining.

Preparation of subcellular fractions. To separate the cytosolic and mitochondrial fractions, the cells were washed with ice-cold PBS. The cells were then lysed using Cell Lysis and Mitochondria Intact buffer (250 mM sucrose, 80 mM KCl and 50 μg/ml digitonin in PBS) on ice for 5 min. The cell suspension was centrifuged at 377 x g for 5 min at 4°C. The supernatant was removed and stored at -20°C as the cytosolic fraction.

Cell cycle analysis. The cells were cultured on 6-well plates at 37°C to reach 70-80% confluence with RPMI-1640 and then treated with vernodalol at various concentrations (25, 50 or 100 μM) for the indicated time. The vernodalol-treated and control cells were harvested by centrifugation for 5 min at 377 x g and room temperature and fixed in 4 ml ice-cold 75% ethanol at 4°C overnight. The cells were stained with 200 µl PI (50 μg/ml; Sigma-Aldrich; Merck KGaA) at 37°C for 10 min and incubated with 20 µl RNase (1 mg/ml; Sigma-Aldrich; Merck KGaA) for removal of RNA in a 37°C water bath for 15-20 min. The cells were then analyzed by flow cytometry (FACS Calibur; BD Biosciences). The results are presented as mean values from three independent experiments.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± standard deviation. The values of half-maximal inhibitory concentration (IC₅₀) were fitted using a nonlinear regression model with a sigmoidal dose response. The values were evaluated
by one-way analysis of variance followed by Tukey's multiple comparison, which was utilized to determine the significance of controls and treated groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Vernodalol inhibits the growth of human acute promyelocytic leukemia cells. The antitumor effects of vernodalol were initially tested using three human APL cell lines, NB4, KG-1a and HL-60. Cells were treated with different concentrations of vernodalol or vehicle (DMSO) for 24, 48 and 72 h, and the cell viability was determined by MTT assay. As shown in Fig. 1, the treatment of three APL cell lines with vernodalol resulted in a dose- and time-dependent attenuation of cell viability. IC\textsubscript{50} values were calculated and are listed in Table I. The results showed that vernodalol inhibits the growth of APL cells in a dose- and time-dependent manner (P<0.01). Among these three APL cell lines, HL-60 was more sensitive to vernodalol compared with NB4 and KG-1a cell lines. Therefore, HL-60 was selected for subsequent experiments.

Vernodalol induces G2/M growth arrest. To examine whether vernodalol inhibited proliferation through the induction of cell cycle arrest, the effect of vernodalol on the cell cycle was assessed. HL-60 cells were treated with different concentrations of vernodalol for 24 h, and the DNA-based cell cycle was analyzed by flow cytometry following PI staining. Vernodalol caused a marked accumulation of cells in the G2/M phase in a dose-dependent manner (Fig. 2A and B). Western blot analysis was performed to examine the effect of vernodalol on possible cell cycle-associated targets, including p21, cyclin B1 and Cdc25. As shown in Fig. 2C and D, vernodalol induced the upregulation of p21 and Cdc25 and the downregulation of cyclin B1 in a dose-dependent manner. These data indicated that vernodalol may induce G2/M cell cycle arrest via cell cycle regulatory molecules in APL cells.

Vernodalol induces apoptosis through the mitochondrial apoptosis pathway. Next, it was examined whether vernodalol had an effect on cell apoptosis. HL-60 cells were stained with Annexin V/PI subsequent to being treated with different concentrations of vernodalol for 24 h and subjected to flow cytometric analysis. As shown in Fig. 3A and B, the proportion of cells in early apoptosis (Annexin V+/PI-; right lower quadrant), as well as late apoptosis (Annexin V+/PI+; right upper quadrant), was increased with the different concentrations of vernodalol in a dose-dependent manner. These data indicated that vernodalol is able to induce cell apoptosis in human APL cells.

To elucidate the mechanism by which vernodalol induced cell apoptosis, apoptosis-associated proteins were examined following treatment with vernodalol. The results indicated that vernodalol caused cleavage of caspase-9 and -3 in HL-60 cells (Fig. 3C and D). In addition, cleaved PARP was also detected, which is a marker of cells undergoing apoptosis (Fig. 3C and D). This suggested that the caspase cascade and PARP inactivation are involved in vernodalol-mediated apoptosis. As demonstrated in Fig. 4, vernodalol downregulated the expression of Bcl-2 and Mcl-1 but upregulated Bim, Bax and Bad expression in a dose-dependent manner. In addition, it was investigated whether the mitochondrial apoptosis pathway was involved in vernodalol-induced apoptosis. The release of cytochrome c and Smac into the cytosol was measured following cell treatment with vernodalol. Vernodalol caused a significant increase in the expression of cytochrome c and Smac/DIABLO in the cytosol (Fig. 4B and C). These results indicated that vernodalol induces cell apoptosis through the mitochondrial apoptosis pathway in APL cells.

Vernodalol inhibits the PI3K/Akt signaling pathway. It has been reported that the inhibition of the PI3K/Akt/mTOR signaling

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Table I. IC\textsubscript{50} values of vernodalol on NB4, KG-1a and HL-60 cell lines.

| Cell lines | 24 h       | 48 h       | 72 h       |
|------------|------------|------------|------------|
| NB4        | 65.72±4.4  | 36.6±4.5   | 17.06±4.0  |
| KG-1a      | 76.4±1.5   | 42.53±4.5  | 23.78±2.9  |
| HL-60      | 67.83±2.9  | 38.36±3.4  | 16.43±3.5  |

IC\textsubscript{50}, half-maximal inhibitory concentration.
pathway has a vital role in the inhibition of tumors (17). Therefore, western blot analysis was performed to investigate whether the PI3K/Akt/mTOR signaling pathway was involved in the vernodalol-mediated anticancer effects. The results demonstrated that the phosphorylation of Akt at Ser473 as well as the expression of mTOR, a well-known downstream target of Akt, were downregulated in cells treated with vernodalol in a dose-dependent manner (Fig. 5). Furthermore, there were little changes in the levels of PI3K-p110α and total Akt. Additionally, PTEN, a key negative regulator of the PI3K/Akt pathway, was upregulated in the treated cells (Fig. 5) compared with the untreated cells. These results provide compelling preliminary data that vernodalol may exert its antitumor function through inhibition of the PI3K/Akt/mTOR signal pathway.

Discussion

APL is a distinct subtype of acute leukemia (9). Patients with APL exhibit a tendency to bleed severely and prognosis is poor with a fatal course in only weeks (5). Although the clinical outcomes of refractory, relapsed and newly diagnosed patients with APL have been greatly improved, the mortality rate remains high (18). The present study demonstrated that vernodalol isolated from C. anthelminticum exerted antitumor effects in human APL cells. Vernodalol suppressed growth of human APL cells by inducing apoptosis through the mitochondrial pathway and causing cell cycle arrest in the G2/M phase. In addition, the results suggested that the strong cytokinetic effects of vernodalol were in part mediated via inhibition of the PI3K/Akt signaling pathway.

Vernodalol is a sesquiterpene lactone isolated from the seeds of C. anthelmintica (19). To date, a limited number of studies, which investigate the anti-cancer effects of vernodalol in human breast cancer cells and skin cancer models have been performed (10,20). These studies demonstrated the cytotoxic activity of vernodalol on melanoma and ovarian cancer cell lines, and human nasopharyngeal carcinoma (21,22). However, the effect and the precise mechanism of vernodalol on human APL cells, remains unclear. To the best of our knowledge, this is the first study investigating the function and mechanism of vernodalol in human APL cells.

Cell cycle dysregulation contributes to tumor initiation and progression. Therefore, cell cycle arrest has become the major focus of anticancer treatments (23). The majority of chemotherapeutic agents cause cell cycle arrest either at the G0/G1 or G2/M stage (24,25). In the present study, downregulation of cyclin B1 expression and upregulation of p21 and Cdc25, was observed following vernodalol treatment. p21, which belongs to the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors, binds to and inhibits the kinase activity of CDK2 and CDK1, leading to growth arrest at specific stages of the cell cycle (26). Cdc25C triggers G2 progression to mitosis by dephosphorylation of the cyclin B1/Cdk1 complex (26). Cyclin B1 is also a key cell cycle regulator of the G2/M phase transition, and the prototypical cyclin B1 reaches maximum levels in G2 when it enters the nucleus to form a complex
with Cdk1 in a phosphorylation-dependent manner (27). It has been reported that the overexpression of cyclin B1 causes uncontrolled cell growth and may even promote the malignant transformation required for the initiation of mitosis (28). Taken
together, the present data suggest a novel cell cycle regulating property of vernodalol via G2-phase arrest in human APL cells.

Apoptosis, a complex program of cell death, is regulated by numerous molecular signaling pathways (29). A study has shown that the mitochondrial pathway performs a key role in the apoptotic process (10). In this pathway, there is an increase in the release of apoptogenic factors (cytochrome c and Smac/DIABLO) from the outer mitochondrial membrane space into the cytosol due to changes in the mitochondrial membrane potential (Dym). The Bcl-2 family is a major regulator of programmed cell death (30). A previous study reported that Bcl-2 can block cell death in mitochondria by inhibiting the apoptosis-associated release of cytochrome c from the mitochondria (30). Results of the present study indicated a
decrease in the levels of anti-apoptotic proteins Bcl-2 and Mcl-1 and an increase in pro-apoptotic proteins Bim, Bax and Bad following treatment with vernodalol (Fig. 4A). Furthermore, the levels of cytochrome c and Smac/DIABLO were increased in the cytosol following treatment with vernodalol. Therefore, the present findings indicated that treatment with vernodalol induces apoptosis in a manner that is dependent on activation of the mitochondrial apoptosis pathway.

The PI3K/Akt signaling pathway is a cell survival pathway that is important for normal cell growth and proliferation (31). Numerous studies have demonstrated that the PI3K/Akt signaling pathway is involved in various cellular functions,
including protein synthesis, cell cycle progression, cell survival, apoptosis and angiogenesis (31,32). The amplification of PI3K/Akt signal transduction is the main cause of cellular growth, and the aberrant activation of PI3K/Akt due to genetic variation in key genes has been observed in several types of cancer, including leukemia and esophageal cancer (33,34). It has been reported that the PI3K/Akt signaling axis is constitutively activated in APL, and the addition of PI3K and mTOR inhibitors to induction treatment regimens may provide therapeutic benefits for APL (33,35). Akt is one of the major downstream effectors of PI3K (36). Upon PI3K activation, Akt is translocated to the inner membrane via its pleckstrin homology domain where it is phosphorylated by 3-phosphoinositide-dependent kinase-1 on its activation loop (37). This Akt modification is sufficient to activate the mTOR complex 1, which was originally identified as a crucial component of insulin receptor intracellular signaling (38). PTEN, a tumor suppressor phosphatase and tensin homolog, may antagonize PI3K/Akt signaling (39). PTEN was identified as a frequently mutated gene in numerous types of tumors, particularly endometrium, skin, brain and prostate (40). A study has also revealed that the downregulation of PI3K, Akt and mTOR and increased PTEN gene expression induces apoptosis and inhibits cell cycle progression in the HL-60 cell line (33). Therefore, the abrogation of PI3K or Akt function may be crucial for APL therapy. In the present study, western blot analysis demonstrated that vernodalol attenuated the phosphorylation of Akt at Ser473 and the expression of the downstream target mTOR and increased the expression of tumor suppressor PTEN (Fig. 5A) in a dose-dependent manner in human APL cells. These results suggest that vernodalol may induce apoptosis and inhibit cell cycle G2/M arrest by suppressing the PI3K/Akt signaling pathway. However, further investigation is required to validate this hypothesis.

In conclusion, to the best of our knowledge, the present study reported for the first time that vernodalol, an active compound isolated from Centratherum anthelminticum, has a potential therapeutic function in human APL cells. Vernodalol induces cell apoptosis and G2/M growth arrest. In addition, the present findings also highlighted the therapeutic value of the repression of the PI3K/Akt pathway by vernodalol in human APL cells. Therefore, vernodalol may be a potential candidate used for the treatment of APL, subsequent to further investigation.

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