

CD-200 induces apoptosis and inhibits Bcr-Abl signaling in imatinib-resistant chronic myeloid leukemia with T315I mutation

ZHENGHUAN FANG^{1*}, KYUNG HEE JUNG^{1*}, HONG HUA YAN¹, SOO JUNG KIM¹, MI KWON SON¹, MARUFA RUMMAN¹, HYUNSEUNG LEE¹, KI WOON KIM², HYE-DONG YOO² and SOON-SUN HONG¹

¹Department of Biomedical Sciences, College of Medicine, Inha University, Incheon 400-712;

²Chodang Pharmaceutical Research Institute, Seoul 152-860, Republic of Korea

Received February 23, 2015; Accepted April 9, 2015

DOI: 10.3892/ijo.2015.2994

Abstract. Chronic myeloid leukemia (CML) is characterized by a constitutively active Bcr-Abl tyrosine kinase. Although Imatinib has been proven to be an effective drug against CML, its resistance has been observed with disease relapse due to T315I predominant point mutation. *Liriodendron tulipifera* L., one of the fastest growing hardwood tree species, exerts anti-oxidant activity and anti-inflammatory effects. However, its anticancer effect has been minimally reported. In this study, we extracted CD-200 from *Liriodendron tulipifera* L. and investigated its effect on cell survival or apoptosis in CML cells with Bcr-Abl/T315I (BaF3/T315I) as well as wild-type Bcr-Abl (BaF3/WT). CD-200 inhibited cell proliferation in the BaF3/WT cells, and also in the BaF3/T315I cells with Imatinib resistance. Moreover, it strongly inhibited Bcr-Abl signaling pathways in a dose-dependent manner. Also, it significantly increased the sub-G₁ phase and the expression of cleaved PARP and caspase-3, as well as the TUNEL-positive apoptotic cells. In addition, we observed that CD-200 induced apoptosis with a loss of mitochondrial membrane potential by decreasing the expression of Mcl-1 and survivin. Furthermore, CD-200 showed a significant inhibition in tumor growth, compared to Imatinib in BaF3/T315I mouse xenograft models. Taken together, our study demonstrates that CD-200 exhibits apoptosis induction and anti-proliferative effect by blocking the Bcr-Abl signaling pathways in the Bcr-Abl/T315I with resistance to Imatinib. We suggest that CD-200 may be a natural product to target Bcr-Abl and overcome Imatinib resistance in CML patients.

Introduction

Chronic myelogenous leukemia (CML) is one of the neoplasms characterized by abnormally elevated white blood cells (WBCs) (1). The incidence of CML in the United States is ~5,000 cases per year, and its prevalence has been increasing annually since 2000, owing to the low annual mortality rate of 1-2% (2). CML has a pivotal place in oncology because >95% of affected individuals express fusion Bcr-Abl protein, a genetic rearrangement formed by reciprocal translocation between chromosomes 9 and 22, which results in a shortened 22q-the Philadelphia chromosome (Ph) (3). The subsequent deregulation of Bcr-Abl leads to enhanced proliferation, resistance to apoptosis, and altered adhesion through hyper-activation of various signaling pathways, including MAPK/Erk and PI3K/Akt (4). The universal presence of Bcr-Abl fusion protein in CML patients has led to the development of small molecule kinase inhibitors to target Bcr-Abl.

Imatinib, the first generation powerful tyrosine kinase inhibitor (TKI), revolutionized the treatment of CML (5). Imatinib has demonstrated excellent treatment for CML due to a cumulative complete cytogenetic response (CCyR) rate of 87% and the projected overall and progression-free survival rates of 89% in a newly diagnosed patient with CML-chronic phase (CML-CP) (6). Unfortunately, resistance to Imatinib is encountered, and an increasing number of recent studies have documented a lack of patient response to Imatinib (7,8). Imatinib resistance might be due to amplification and point mutation in a kinase domain of Bcr-Abl (9,10).

More than 40 kinds of different point mutations have been classified to be related with clinical resistance to Imatinib, and especially 20% of all the point mutations are identified to be a stubborn point mutation, namely the T315I point mutation, which is an amino acid transformation in the Abl 315th position (11). Although, the second generation Bcr-Abl TKIs, such as Nilotinib and Dasatinib, have been developed and circumvented several types of point mutations (E255K, M351T), those have not overcome T315I mutation as well (12,13). These TKIs are not curative since most patients who discontinue therapy due to drug resistance will exhibit rapid progression. To overcome this resistance, more effective tyrosine kinase inhibitors are being developed, however, drug resistance and side effects of new agents are still important issues to be

Correspondence to: Professor Soon-Sun Hong, Department of Biomedical Sciences, College of Medicine, Inha University, 3-ga, Sinheung-dong, Jung-gu, Incheon 400-712, Republic of Korea
E-mail: hongss@inha.ac.kr

*Contributed equally

Key words: CD-200, Imatinib, Bcr-Abl, T315I, chronic myeloid leukemia

considered. Therefore, the search for other novel targets and new strategies for the management of CML is urgent.

Plant-derived natural products play an important role in the cure of various diseases. Numerous natural products are under investigation for their clinical efficacy in the prevention and treatment of a wide array of diseases including cancer (14,15). Among these, Yellow poplar or tulip tree, *Liriodendron tulipifera* L. (Magnoliaceae), is a fast growing native timber tree with a tall and straight trunk in North America (16). The bark of *Liriodendron tulipifera* L. was widely used by the Native Americans as a tonic, stimulant, and its diaphoretic properties have been considered effective in treating chronic rheumatism, dyspepsia and avian malaria (17). To date, the phytochemical investigation of *Liriodendron tulipifera* L. has yielded numerous constituents, including antiplasmodial alkaloids, various bioactive lignans, and cytotoxic sesquiterpene lactones (18-21).

A recent study has reported that partenolide, one of the sesquiterpene lactones isolated from *Liriodendron tulipifera* L. and its semi-synthetic derivatives, showed a cytotoxic effect in leukemia cells (22). These facts imply that the extract of *Liriodendron tulipifera* L. has the potential to be an anticancer agent in leukemia. For this reason, *Liriodendron tulipifera* L., a rich source of metabolites with a-methylene- γ -lactone moiety, was chosen to be investigated for anti-leukemic potential. In this study, we extracted CD-200 with rich sesquiterpene lactones from *Liriodendron tulipifera* L. and investigated its anticancer effect and mechanism of action in BaF3/T315I or BaF3/WT leukemic cells. Our present study demonstrated that CD-200 inhibited the Bcr-Abl pathway and induced apoptosis in BaF3/WT cells, and also T315I-mutated Bcr-Abl cells with resistance to Imatinib.

Materials and methods

Extraction of CD-200. Trees of *Liriodendron tulipifera* L., yellow poplar (~0.9-1.4 meters in height), were collected from Kangjin, Chonnam Province, Korea. Air-dried trees (1.0 kg) were chopped off before extraction. The chopped plant materials were exhaustively extracted three times with either ethyl acetate or ethanol. Each extract was separately filtered and concentrated by a rotary evaporator. The resulting concentrates were completely dried via freeze dryer to yield brownish extract (11.3 g). In order to isolate the active ingredient, sesquiterpene lactones, including epi-Tulipinolide from ethyl acetate or ethanol extract, further reversed-phase high-performance liquid chromatography (HPLC) with a stepwise gradient solvent system from H₂O to methanol was performed.

Cells and materials. The BaF3/WT and BaF3/T315I cells were provided by Dr M. Deininger (Huntsman Cancer Institute, Salt Lake City, UT, USA). The cells expressed wild-type Bcr-Abl and Bcr-Abl with the T315I mutation, respectively. BaF3/WT and BaF3/T315I cells were grown in Roswell Park Memorial Institute Medium-1640 (RPMI-1640), containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. RPMI-1640, FBS, and penicillin/streptomycin were purchased from Gibco (Grand Island, NY, USA). Imatinib was purchased from LC Laboratories (Woburn, MA, USA), and sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-

5-carboxanilide inner salt (XTT) assays were purchased from WelGene Inc. (Daegu, Korea).

Cell viability assay. Cell viability of corresponding compounds was determined by XTT assay. The cells were seeded and treated onto 96-well plates at a density of 1×10^3 cells per well and incubated at 37°C for 48 h. The cells were then treated with either CD-200 or Imatinib at the indicated concentrations (0.1-10 μ g/ml) or (0.01-10 μ M), respectively. Then, 10 μ l of XTT labeling mixture [1 ml of XTT/20 μ l of phenazinemethosulfate (PMS)] was added to each well. After incubation for 4 h, optical density (OD) was determined using a microplate reader by measuring the absorbance at wavelengths 540 nm and 620 nm. The absorbance rates for each well were calculated as $OD_{540} - OD_{620}$.

Western blotting. After the cells were treated with various concentrations of either CD-200 or Imatinib and incubated at 37°C for various times, they were collected and washed with cold phosphate-buffered saline (PBS). Then, the cells were lysed with a RIPA buffer (Biosesang, Seongnam, Korea) containing protease and phosphatase inhibitor cocktails (GenDEPOT, Barker, TX, USA). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto the nitrocellulose membranes. The blots were immunostained with appropriate primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase. Antibody binding was detected with an enhanced chemiluminescence reagent (Bio-Rad, Hercules, CA, USA). Primary monoclonal antibodies against the following factors were used; p-Bcr-Abl (Tyr⁷⁷), p-CrkI (Tyr²⁰⁷), p-Stat5 (Tyr⁶⁹⁴), Bcr-Abl, CrkI, Stat5, cleave caspase-3, cleaved PARP, survivin, Mcl-1 and β -actin (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Immunofluorescence. BaF3/T315I cells were plated in 48-well plates with RPMI-1640 medium and treated with CD-200 for various concentrations. The cells were then suspended on poly-L-lysine-coated slides, followed by Shandon Cytospin 3 (Akribis Scientific, Cheshire, WA, USA) for 3 min at 1000 rpm. Thereafter, the cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and washed twice with PBS. The cells were blocked in 5% horse and goat serums in PBS for 1 h at room temperature, then incubated in a humidified chamber at 4°C overnight with p-Bcr-Abl (Tyr⁷⁷) antibody (Cell Signaling Technology). After washing twice with PBS, the cells were incubated with rabbit tetramethyl rhodamine isothiocyanate (TRITC) secondary antibody (Dianova, Germany) for 1 h at room temperature. They were also stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. The slides were then washed twice with PBS and covered with Dako (Carpinteria, CA, USA) before viewing with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Moreover, after deparaffinization, immunostaining was performed, using 8- μ m-thick sections of the tumor samples. The tissue sections were then blocked with a normal goat or horse serum (Vector Laboratories, Burlingame, CA, USA) for

1 h, and were incubated at 4°C overnight with the primary antibody. After washing twice with PBS, the cells were incubated with the rabbit TRITC secondary antibody for 1 h at room temperature. They were also stained with DAPI to visualize the nuclei. The slides were then washed twice with PBS and covered with Dako before viewing with a confocal laser scanning microscope.

Cell cycle analysis. BaF3/T315I cells were plated in 10-cm dishes with an RPMI-1640 medium and treated with CD-200 at the indicated concentrations (0-5 µg/ml). The cells were collected and fixed in cold 70% ethanol at -20°C overnight. After washing with PBS, the cells were subsequently stained with 50 µg/ml propidium iodide (PI) and 100 µg/ml RNase A for 30 min at room temperature in the dark; then a flow cytometric analysis was performed to determine the percentage of cells in specific sub-G₁ phases, using a FACS Calibur flow cytometry (BD Biosciences, San Jose, CA, USA).

TUNEL staining. BaF3/T315I cells were plated in 48-well plates with RPMI-1640 medium and treated with various concentrations of CD-200. The cells were then suspended on poly-L-lysine-coated slides, followed by Shandon Cytospin 3 for 3 min at 1000 rpm. They were then fixed in 4% PFA for 15 min at room temperature and washed twice with PBS. The terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was subsequently performed using a TUNEL kit (Merck Millipore, Temecula, CA, USA) in accordance to the manufacturer's instructions.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential (MMP, $\Delta\psi$) was assessed using the Mitochondrial Membrane Potential Detection kit (BD Biosciences). BaF3/T315I cells were then treated with various concentrations of CD-200 for 8 h. The cells were incubated with JC-1 working solution at 37°C. After washing two times, the cells were suspended in 0.5 ml of 1X assay buffer prior to flow cytometry. The results were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Analysis of cytochrome c localization. BaF3/T315I cells were treated with various concentrations for 8 h. To label the mitochondria, the cells were incubated with 500 nM mitochondrion-specific dye (MitoTracker® Green FM; Molecular Probes Inc., Eugene, OR, USA) for 45 min at 37°C prior to fixation. The cells were then suspended on poly-L-lysine-coated slides, followed by Shandon Cytospin 3 for 3 min at 1000 rpm. They were then fixed in 4% PFA for 15 min at room temperature and washed with PBS. The cells were incubated at 4°C overnight with cytochrome c antibody (Santa Cruz Biotechnology). After washing twice with PBS, the cells were incubated with mouse TRITC secondary antibody (Dianova). The cells were also stained with DAPI to visualize the nuclei. The slides were then washed twice with PBS and covered with Dako before viewing with a confocal laser scanning microscope.

Tumor xenograft study. All animal experiments were performed in accordance to the guidelines of the INHA Institutional Animal Care and Use Committee (INHA IACUC) at Inha University Medical School. The cells were

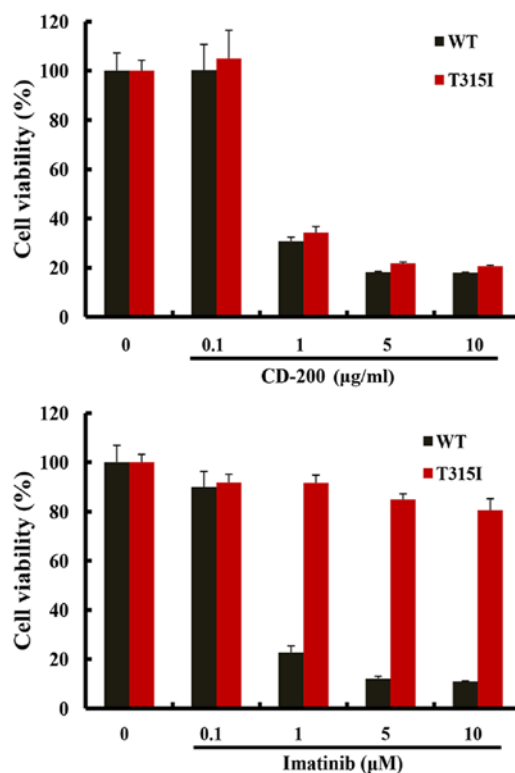


Figure 1. Effect of CD-200 on the proliferation of leukemia cells. Cytotoxic effects of leukemia cells (BaF3/WT and BaF3/T315I cells) treated with CD-200 or Imatinib were measured using XTT assay. Cells were seeded in 96-well culture plates, and were treated with various concentrations of CD-200 or Imatinib. Cells were subjected to XTT assay following incubation for 48 h. Data are presented as the mean \pm SD from triplicate wells.

harvested and mixed in PBS (200 µl/mouse). Six weeks old male BALB/c nude mice (Orient Bio, Seoul, Korea) were inoculated with 1×10^5 cells in the flank. When the tumor size reached approximately 50-100 mm³, they were randomly divided into 3 groups, with 5 mice in each. Then, these mice were given CD-200 (50 mg/kg) or Imatinib (50 mg/kg) intraperitoneally once a day for 11 days, and the control group was fed vehicle. The tumor size was measured every 2 days, and it was calculated using the formula, $0.5 \times \text{length} \times \text{width}^2$.

Immunohistochemistry. The tissue sections were blocked with normal goat or horse serum (Vector Laboratories) for 1 h, and incubated at 4°C overnight in 1:50 dilutions of p-Bcr-Abl, Ki-67, and cleaved caspase-3 (Cell Signaling Technology). The sections were then incubated with biotinylated secondary antibodies (1:100) for 1 h. The sections were visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories), which were then washed in PBS and developed with a diaminobenzidine tetrahydrochloride substrate for 15 min, then counterstained with hematoxylin. At least 3 random fields of each section were examined at x400 magnification and analyzed using a computer image analysis system (Media Cybernetics, Rockville, MD, USA).

Statistical analysis. Data were expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using ANOVA and unpaired Student's t-tests. Statistical significance was set to $p < 0.05$.

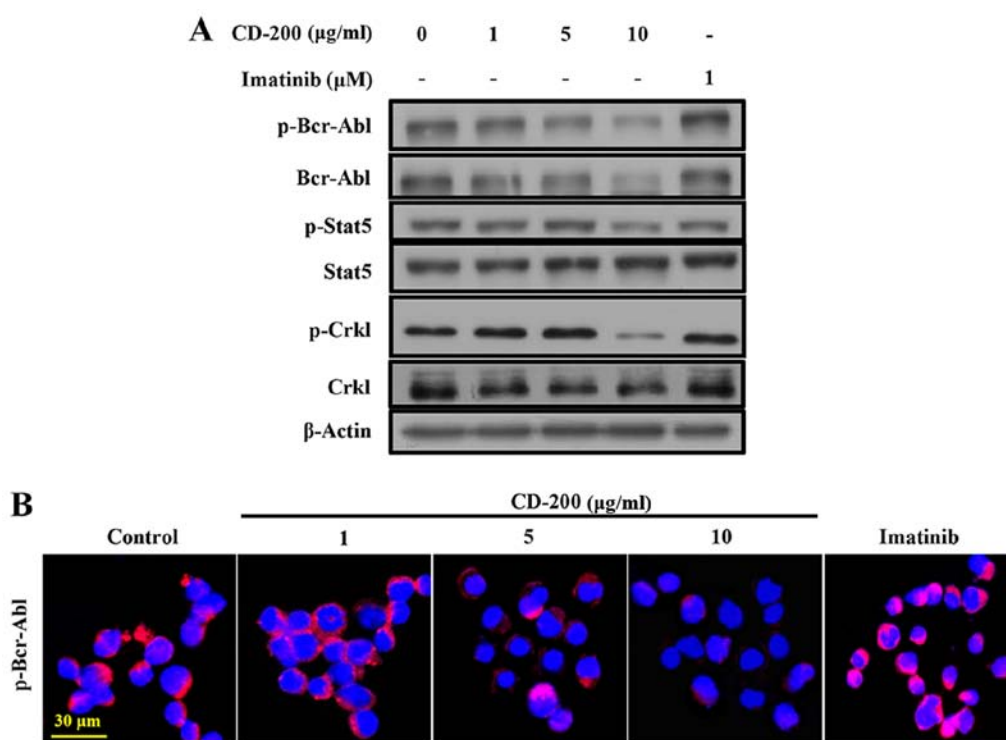


Figure 2. Effect of CD-200 on the Bcr-Abl signaling pathway in BaF3/T315I cells. (A) Cells were treated with CD-200 (1–10 μg/ml) or Imatinib (1 μM) for 2 h. Western blot experiments for Bcr-Abl, Crk1 and Stat5, and the respective phosphorylated proteins were performed with cell lysates. (B) After BaF3/T315I cells were treated with CD-200 for 3 h, p-Bcr-Abl level was detected by confocal fluorescent microscopy (x400 magnification).

Results

CD-200 inhibits the proliferation of BaF3/WT and BaF3/T315I cells. XTT assays were performed to evaluate the effect of CD-200 on the growth of BaF3/WT and BaF3/T315I cells. The cells were treated with various concentrations of CD-200 and Imatinib for 48 h. As shown in Fig. 1, both CD-200 and Imatinib treatments reduced the cell viability of BaF3/WT cells; the IC_{50} values were about 0.6 μg/ml for CD-200 and 0.4 μM for Imatinib. In addition, CD-200 significantly reduced the cell viability of BaF3/T315I cells ($IC_{50} \approx 0.8$ μg/ml), while Imatinib had little effect. These data suggest that CD-200 exhibits the potent inhibitory activity in BaF3 cells, expressing Bcr-Abl T315I mutation resistance to Imatinib, as well as wild-type Bcr-Abl leukemic cells.

CD-200 inhibits Bcr-Abl signaling pathways in BaF3/T315I cells. In order to assess the effects of CD-200 on the inhibition of Bcr-Abl activity, phosphorylation of Bcr-Abl and its respective downstream signals, Crk1 and Stat5, were measured by western blotting. As shown in Fig. 2A, CD-200 strongly inhibited the phosphorylation of Bcr-Abl (Tyr⁷⁷) in BaF3/T315I cells. Likewise, the phosphorylation levels of Crk1 (Tyr²⁰⁷) and Stat5 (Tyr⁶⁹⁴) were effectively suppressed. In contrast, Imatinib did not alter the phosphorylation levels of Bcr-Abl, Crk1, and Stat5 in BaF3/T315I cells. The expression of p-Bcr-Abl was confirmed by confocal fluorescent microscopy (Fig. 2B).

CD-200 induces apoptotic cell death in BaF3/T315I cells. In order to investigate whether the anticancer effect of CD-200

in BaF3/T315I cells was associated with the induction of apoptosis, we performed several cell-based apoptosis assays. We first identified the nuclear morphology on 12 h after treatment with CD-200 by using TUNEL apoptosis assay kits. As a result, CD-200-treated cells were presented with more prominent DNA fragmentation in comparison to the control group (Fig. 3A). Next, we assessed the cell cycle distribution by a flow cytometric analysis. After 24 h treatment with CD-200, the cells were collected and stained with PI, then analyzed by FACS. As shown in the Fig. 3B, CD-200 increased the number of cells in the sub- G_1 phase, associated with early apoptosis without changes of the cell cycle arrest (Fig. 3B). In support of apoptotic effect of CD-200, we observed that CD-200 also increased cleaved caspase-3 positive cells by FACS (Fig. 3C).

CD-200 induces mitochondria-dependent apoptosis in BaF3/T315I cells. To gain further insight into the mechanism underlying apoptosis induced by CD-200, we examined the mitochondrial potential change, which plays an important role in the regulation of apoptosis. Since a loss of MMP induces the transition of mitochondrial permeability and release of cytochrome c from the mitochondria to cytosol (23), we measured the MMP and cytochrome c release in CD-200-treated BaF3/T315I cells. As shown in Fig. 4A, CD-200 significantly reduced the fluorescence intensity reflecting MMP, while no changes were observed in the Imatinib-treated group. Moreover, we observed that the treatment of CD-200 increased the release of cytochrome c by immunostaining (Fig. 4B). In addition, CD-200 inhibited the expression of mitochondria-mediated protein families, such as Mcl-1, and survivin (Fig. 4C) along with that of cleaved PARP. These results indicated that CD-200

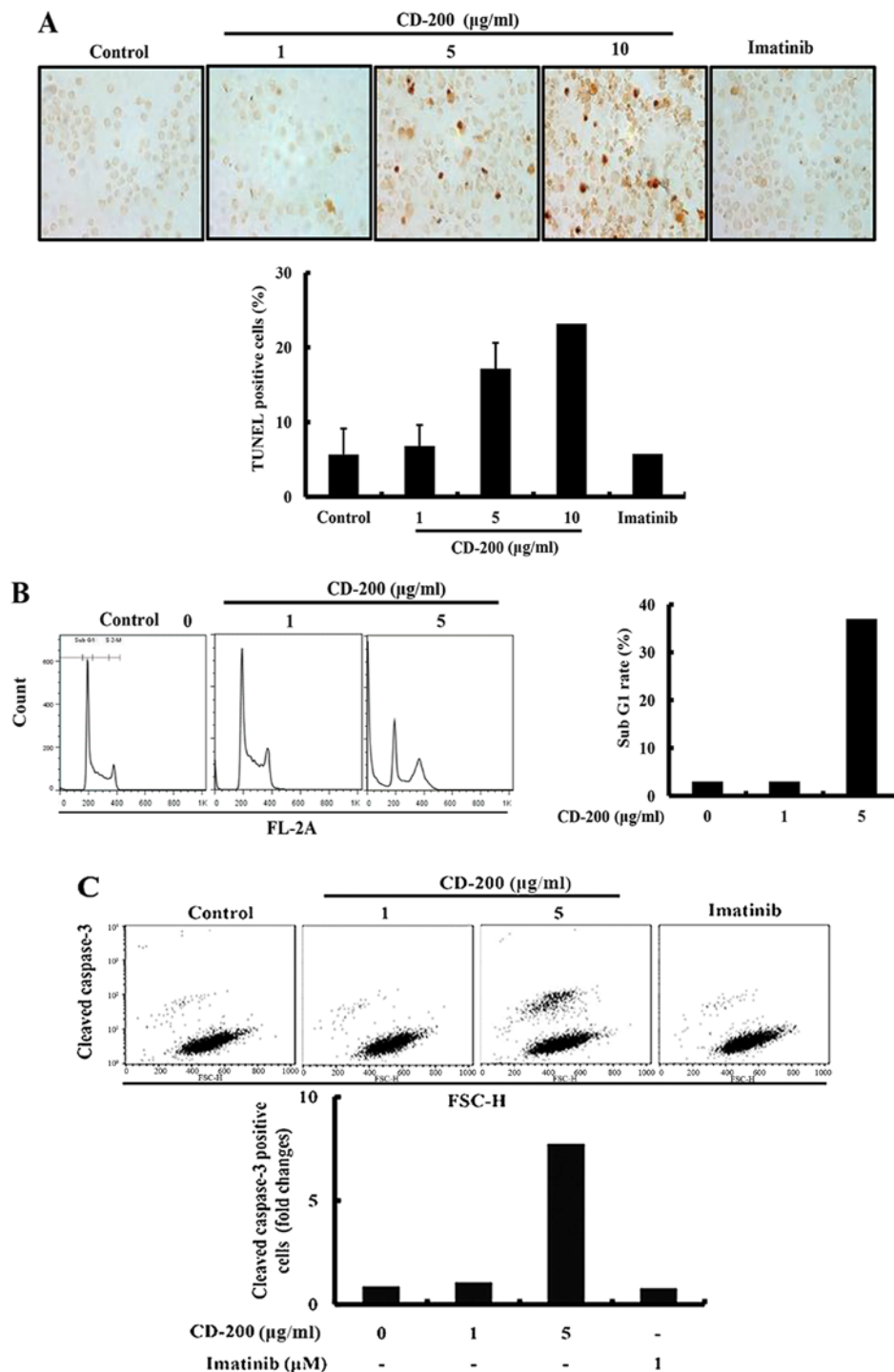


Figure 3. Effect of CD-200 on apoptosis of BaF3/T315I cells. (A) Cells were treated with CD-200 (1-10 $\mu\text{g/ml}$) or Imatinib (1 μM) for 14 h and were performed with TUNEL assay (x400 magnification). (B) To observe early apoptosis, cells were incubated with CD-200 (1 and 5 $\mu\text{g/ml}$). Control and treated cells were collected, stained with PI, and analyzed by flow cytometry. (C) Cells were incubated with CD-200 or Imatinib for 12 h and cleaved caspase 3-positive cells were determined by FACS analysis, and then were counted and quantified. Data are represented as the mean \pm SD.

induced apoptosis through the mitochondria-mediated intrinsic pathways in BaF3/T315I cells.

CD-200 inhibits tumor growth in mouse xenograft models. We extended our study to an *in vivo* mouse xenograft model. After inoculation with BaF3/T315I cells, mice were intraperitoneally injected with CD-200, at doses of 50 mg/kg and Imatinib, once a day for 11 days. CD-200 potently inhibited the progression of tumor growth, which became more noticeable and significant

on day 11 as compared to the control group, whereas Imatinib treatment did not show significant anticancer effect in this BaF3/T315I cell xenograft model (Fig. 5A). No significant changes in body weight or adverse effect were observed in any of the groups (data not shown). To further confirm whether CD-200 inhibits tumor growth through the induction of apoptosis and inhibition of proliferation, we identified the expression of cleaved caspase-3 and Ki-67 in tumor tissues. As expected, CD-200-treated tumor showed an increased expression of

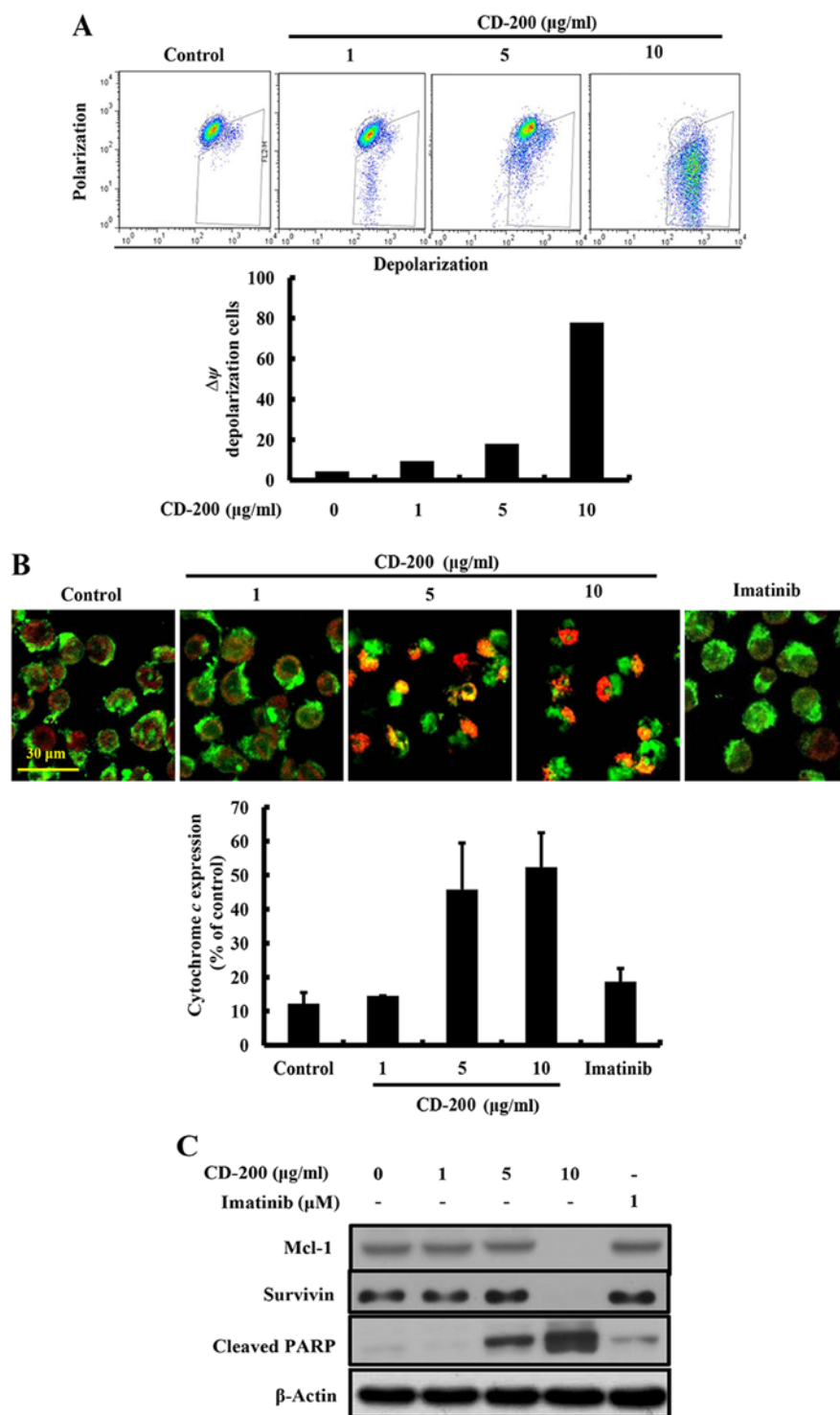


Figure 4. Effect of CD-200 on mitochondria-related apoptosis of BaF3/T315I cells. (A) Mitochondrial damage by CD-200 treatment was investigated by JC-1 assay. When CD-200 (1–10 $\mu\text{g/ml}$) was treated for 8 h, the mitochondria potential was changed, inducing apoptosis of BaF3/T315I cells. (B) BaF3/T315I cells were treated with CD-200 (1–10 $\mu\text{g/ml}$) and Imatinib (1 μM) or 8 h and were stained with mitotracker and cytochrome c. Localization of cytochrome c in the cytosol by CD-200 was analyzed with a confocal fluorescent microscopy (x400 magnification). For quantification of cells with diffuse cytochrome c staining, we analyzed the percentage of cells that showed cytochrome c release. (C) The expression of Mcl-1, survivin and cleaved PARP was assayed by western blotting in BaF3/T315I cells. Cells were treated with CD-200 or Imatinib for 12 h.

cleaved caspase-3 and decreased Ki-67 expression compared to the control and Imatinib groups (Fig. 5B). Moreover, the treatment with CD-200 decreased the phosphorylation of p-Bcr-Abl (Fig. 5B). Taken together, these results demonstrate that CD-200 has antitumor potency in the mouse xenograft model bearing BaF3/T315I cells.

Discussion

For over 40 years, natural products have played a very important role in cancer chemotherapy, either as unmodified or synthetically modified forms (24). For instance, plant-derived compounds are widely used as anticancer agents, such as bisin-

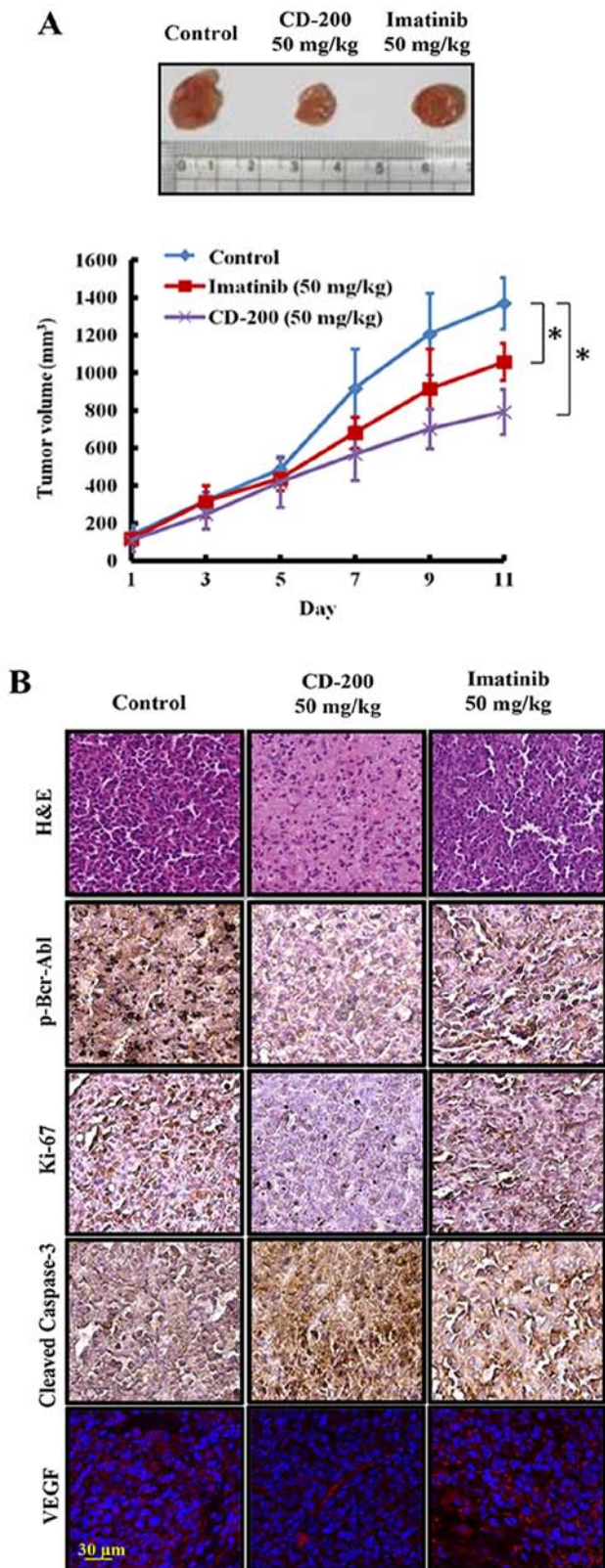


Figure 5. *In vivo* anticancer effect of CD-200 in a mouse xenograft model. (A) Representative isolated tumors and tumor growth curve in BaF3/T315I mouse xenografts. All mice were subjected to implantation in the flank by a subcutaneous injection of BaF3/T315I (1×10^5 cells/200 μ l PBS). CD-200 (50 mg/kg) or Imatinib (50 mg/kg) was intraperitoneally injected once a day for 11 days. Tumor size was measured every 2 days. Data are presented as the mean \pm SD; (n=5). * $p < 0.01$ as compared to the control. (B) Effect of CD-200 in tumor tissues from the mouse xenograft model. Tumors were excised and processed for immunohistochemistry to detect p-Bcr-Abl, Ki-67 and cleaved caspase-3 including H&E staining. The expression of VEGF was detected by confocal fluorescent microscopy (x400 magnification).

dole (vinca) alkaloids, camptothecins, epipodophyllotoxins, and taxanes (25). The focus in cancer control has been on the search for safer anticancer agents, with higher patient acceptability. Thus, the use of herbal/natural products is more popular over synthetic drugs, and provides alternative treatment options for patients (26,27). Natural products have been investigated for cancer prevention and treatment (27,28). In this study, we extracted CD-200 from *Liriodendron tulipifera* L., with abundant sesquiterpene lactones. Based on a previous study where sesquiterpene lactones, isolated from *Liriodendron tulipifera* L., showed cytotoxic effect in leukemia cells (22), we set out to identify the anticancer effects of CD-200, and its mechanism of action in BaF3/WT and BaF3/T315I leukemia cells with Imatinib resistance. Herein, we report for the first time that CD-200 inhibited the Bcr-Abl signaling pathway, which may lead to the inhibition of cell growth and induction of apoptosis *in vitro* and *in vivo* models of Imatinib-resistant CML.

Imatinib has been used as a major TKI to target the Bcr-Abl tyrosine kinase activity in CML patients for decades (29). However, Imatinib resistance develops over time and is an emerging problem for CML patients. Although, Imatinib strongly inhibits the phosphorylation of tyrosine in the wild-type Bcr-Abl, it does not act on Bcr-Abl with T315I mutations (30). Although the new second-generation Abl kinase inhibitors, such as AMN107, Dasatinib, INNO-406 and PD166326, have been developed, they do not show any positive effects in Imatinib-resistant patients with T315I mutation (12,13,31,32). Thus, it is important to develop alternative treatment strategies. In this study, we aimed to identify effective chemotherapy with the use of natural products against CML cells with T315I-mutant Bcr-Abl that confers resistance to Imatinib. To carry this out, we extracted CD-200 from *Liriodendron tulipifera* L., and investigated whether CD-200 had potent activity in BaF3-expressing wild-type Bcr-Abl and T315I-mutated Bcr-Abl cells. CD-200 strongly inhibited the cell proliferation in both BaF3/WT and BaF3/T315I cells. However, Imatinib failed to inhibit the proliferation of BaF3/T315I cells, since Imatinib did not inhibit cell growth even at a high concentration (10 μ M).

The Bcr-Abl kinase signals affects multiple downstream survival pathways, including Ras/Raf/Mek, PI3K/Akt, and Jak/Stat, which contribute to the pathogenesis of CML (33,34). Especially, Stat5 pathway, a surrogate marker of Bcr-Abl activity in primary CML cells, contributes to leukemic cell proliferation and survival (35). Furthermore, the Stat5 activation is mediated by an adaptor protein, Crkl-like protein (Crkl) (36). In addition, Stat5 activity appears to play a major role in anti-apoptotic and proliferative abilities of Bcr-Abl transformed cells (37). Thus, we investigated whether CD-200 could suppress the Bcr-Abl signaling pathway. As expected, CD-200 inhibited the phosphorylation of Bcr-Abl and the phosphorylation of Bcr-Abl downstream target Stat5 in BaF3/T315I cells. Also, the phosphorylation of Crkl, another Bcr-Abl downstream target, was clearly reduced by CD-200 treatment. On the contrary, Imatinib did not inhibit the phosphorylation of the Bcr-Abl pathways, such as Bcr-Abl, Stat5 and Crkl in BaF3/T315I cells. These results reveal that the decrease of phosphorylation of Crkl and Stat5 by CD-200 indicate an effective inhibition of Bcr-Abl carrying T315I highly resis-

tant mutation, whereas no inhibition of phosphorylation was induced by Imatinib treatment.

Apoptosis is a controlled form of cell death with the ability to contribute to the inhibition of cell growth in cancer cells. To date, the molecular mechanisms by which anticancer drugs induce apoptosis have been reported to involve the activation of various apoptotic signaling or inhibition of survival signaling (38,39). Bcr-Abl is known as a potent cell death inhibitor and inhibits apoptosis, as well as the enhancement of cell proliferation in CML (40,41). Bcr-Abl positive cells continue to signal biochemically to prevent apoptosis induced by chemotherapeutic therapy (42). Previous studies have shown that Bcr-Abl prevents apoptosis through the inhibition of mitochondrial cytochrome c release (41). In keeping with these reports, we examined whether CD-200 has an effect on the mitochondrial membrane potential and induction of apoptosis in BaF3/T315I cells. In this study, we observed that CD-200 increased cytochrome c release and decreased the expression of Mcl-1 and survivin, which has been proposed to bind to caspases to inhibit apoptosis with mitochondria potential-related molecules (43). CD-200 induced an increased expression of cleaved PARP, as well as TUNEL positive apoptotic bodies.

Previously, anticancer effects of several sesquiterpene lactones, a main ingredient of CD-200, isolated from various plants have been reported to modulate various molecular signal transduction pathways (44–46). Especially, Yeh *et al* have reported that sesquiterpene lactones induced apoptosis by increasing caspase-3 through a modulation of the Stat signaling pathway in lung cancer (47). Similarly, CD-200, including sesquiterpene lactones, also induced apoptosis by changing the mitochondria potential in CML cells, although their source were different. These events were supported by *in vivo* results, showing that CD-200 inhibited the tumor growth and induced apoptosis by increasing the expression of cleaved caspase-3 in tumor tissues of xenograft mouse BaF3/T315I cells. In contrast, Imatinib did not change the expression of apoptosis-related molecules *in vitro* and *in vivo*. Considering all the above results, CD-200 induced apoptosis via a mitochondria-dependent pathway in BaF3/T315I cells, suggesting that apoptosis by CD-200 may be accomplished by inhibiting the Bcr-Abl signaling pathways.

In conclusion, our study demonstrated that CD-200 suppressed tumor growth and induced mitochondria-mediated apoptosis by downregulating the Bcr-Abl signaling pathway. Our findings could be considered for future clinical investigations in CML patients with Imatinib resistance. We suggest that CD-200 may have great potential in overcoming T315I mutation-induced Imatinib resistance in patients with CML.

Acknowledgements

This study was supported by Inha University Grant and Medical Research Center (2014009392) funded by MSIP, Korea.

References

1. Savona M and Talpaz M: Chronic myeloid leukemia: changing the treatment paradigms. *Oncology* (Williston Park) 20: 707-711; discussion 712-704, 719, 724, 2006.
2. Huang X, Cortes J and Kantarjian H: Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. *Cancer* 118: 3123-3127, 2012.
3. Kurzrock R, Gutterman JU and Talpaz M: The molecular genetics of Philadelphia chromosome-positive leukemias. *N Engl J Med* 319: 990-998, 1988.
4. Tomoda K, Kato JY, Tatsumi E, Takahashi T, Matsuo Y and Yoneda-Kato N: The Jbl1/COP9 signalosome subcomplex is a downstream mediator of Bcr-Abl kinase activity and facilitates cell-cycle progression. *Blood* 105: 775-783, 2005.
5. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, *et al*: Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 344: 1031-1037, 2001.
6. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, *et al*; IRIS Investigators: Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 355: 2408-2417, 2006.
7. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J and Sawyers CL: Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2: 117-125, 2002.
8. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN and Sawyers CL: Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 293: 876-880, 2001.
9. Al-Achkar W, Wafa A, Moassass F, Klein E and Liehr T: Multiple copies of BCR-ABL fusion gene on two isodicentric Philadelphia chromosomes in an imatinib mesylate-resistant chronic myeloid leukemia patient. *Oncol Lett* 5: 1579-1582, 2013.
10. Hochhaus A, Kreil S, Corbin AS, La Rosée P, Müller MC, Lahaye T, Hanfstein B, Schoch C, Cross NC, Berger U, *et al*: Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 16: 2190-2196, 2002.
11. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, Cowan-Jacob SW, Lee FY, Heinrich MC, Deininger MW, *et al*: In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* 65: 4500-4505, 2005.
12. Martinelli G, Iacobucci I, Soverini S, Palandri F, Castagnetti F, Rosti G and Baccarani M: Nilotinib: A novel encouraging therapeutic option for chronic myeloid leukemia patients with imatinib resistance or intolerance. *Biologics* 1: 121-127, 2007.
13. Shah NP, Tran C, Lee FY, Chen P, Norris D and Sawyers CL: Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 305: 399-401, 2004.
14. Goh SS, Woodman OL, Pepe S, Cao AH, Qin C and Ritchie RH: The red wine antioxidant resveratrol prevents cardiomyocyte injury following ischemia-reperfusion via multiple sites and mechanisms. *Antioxid Redox Signal* 9: 101-113, 2007.
15. Winkelmann I, Diehl D, Oesterle D, Daniel H and Wenzel U: The suppression of aberrant crypt multiplicity in colonic tissue of 1,2-dimethylhydrazine-treated C57BL/6J mice by dietary flavone is associated with an increased expression of Krebs cycle enzymes. *Carcinogenesis* 28: 1446-1454, 2007.
16. Lafon CW: Stand dynamics of a yellow-poplar (*Liriodendron tulipifera* L.) forest in the Appalachian Mountains, Virginia, USA. *Dendrochronologia* 22: 43-52, 2004.
17. Rafinesque CS: Medical Flora or Manual of the Medical Botany of the United States of North America. In two volumes. Philadelphia, 1828.
18. Graziose R, Rathinasabapathy T, Lategan C, Poulev A, Smith PJ, Grace M, Lila MA and Raskin I: Antiplasmodial activity of aporphine alkaloids and sesquiterpene lactones from *Liriodendron tulipifera* L. *J Ethnopharmacol* 133: 26-30, 2011.
19. Li WJ, Lin YC, Wu PF, Wen ZH, Liu PL, Chen CY and Wang HM: Biofunctional constituents from *Liriodendron tulipifera* with antioxidants and anti-melanogenic properties. *Int J Mol Sci* 14: 1698-1712, 2013.
20. Doskotch RW and el-Feraly FS: Antitumor agents. II. Tulipinolide, a new germacranolide sesquiterpene, and constunolide. Two cytotoxic substances from *Liriodendron tulipifera* L. *J Pharm Sci* 58: 877-880, 1969.
21. Moon MK, Oh HM, Kwon BM, Baek NI, Kim SH, Kim JS and Kim DK: Farnesyl protein transferase and tumor cell growth inhibitory activities of lipiferolide isolated from *Liriodendron tulipifera*. *Arch Pharm Res* 30: 299-302, 2007.
22. Neelakantan S, Nasim S, Guzman ML, Jordan CT and Crooks PA: Aminoparthenolides as novel anti-leukemic agents: Discovery of the NF-kappaB inhibitor, DMAPT (LC-1). *Bioorg Med Chem Lett* 19: 4346-4349, 2009.

23. Kaufmann SH and Hengartner MO: Programmed cell death: Alive and well in the new millennium. *Trends Cell Biol* 11: 526-534, 2001.
24. Kinghorn AD, Chin YW and Swanson SM: Discovery of natural product anticancer agents from biodiverse organisms. *Curr Opin Drug Discov Devel* 12: 189-196, 2009.
25. Balunas MJ, Su B, Landini S, Brueggemeier RW and Kinghorn AD: Interference by naturally occurring fatty acids in a noncellular enzyme-based aromatase bioassay. *J Nat Prod* 69: 700-703, 2006.
26. Zhao J: Nutraceuticals, nutritional therapy, phytonutrients, and phytotherapy for improvement of human health: A perspective on plant biotechnology application. *Recent Pat Biotechnol* 1: 75-97, 2007.
27. Hong SW, Jung KH, Lee HS, Choi MJ, Son MK, Zheng HM and Hong SS: SB365 inhibits angiogenesis and induces apoptosis of hepatocellular carcinoma through modulation of PI3K/Akt/mTOR signaling pathway. *Cancer Sci* 103: 1929-1937, 2012.
28. de Oliveira LZ, Farias IL, Rigo ML, Glanzner WG, Gonçalves PB, Cadoná FC, Cruz IB, Farias JG, Duarte MM, Franco L, *et al*: Effect of *Uncaria tomentosa* extract on apoptosis triggered by oxaliplatin exposure on HT29 cells. *Evid Based Complement Alternat Med* 2014: 274786, 2014.
29. Deininger M, Buchdunger E and Druker BJ: The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 105: 2640-2653, 2005.
30. O'Hare T, Corbin AS and Druker BJ: Targeted CML therapy: Controlling drug resistance, seeking cure. *Curr Opin Genet Dev* 16: 92-99, 2006.
31. Jabbour E, Cortes J and Kantarjian H: Nilotinib for the treatment of chronic myeloid leukemia: An evidence-based review. *Core Evid* 4: 207-213, 2009.
32. Quintás-Cardama A, Kantarjian H and Cortes J: Flying under the radar: The new wave of BCR-ABL inhibitors. *Nat Rev Drug Discov* 6: 834-848, 2007.
33. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE and McCubrey JA: JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* 18: 189-218, 2004.
34. Kirchner D, Duyster J, Ottmann O, Schmid RM, Bergmann L and Munzert G: Mechanisms of Bcr-Abl-mediated NF-kappaB/Rel activation. *Exp Hematol* 31: 504-511, 2003.
35. Klejman A, Schreiner SJ, Nieborowska-Skorska M, Slupianek A, Wilson M, Smithgall TE and Skorski T: The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J* 21: 5766-5774, 2002.
36. Hemmeryckx B, van Wijk A, Reichert A, Kaartinen V, de Jong R, Pattengale PK, Gonzalez-Gomez I, Groffen J and Heisterkamp N: Crkl enhances leukemogenesis in BCR/ABL P190 transgenic mice. *Cancer Res* 61: 1398-1405, 2001.
37. Hoelbl A, Schuster C, Kovacic B, Zhu B, Wickre M, Hoelzl MA, Fajmann S, Grebien F, Warsch W, Stengl G, *et al*: Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med* 2: 98-110, 2010.
38. Cabot MC, Giuliano AE, Han TY and Liu YY: SDZ PSC 833, the cyclosporine A analogue and multidrug resistance modulator, activates ceramide synthesis and increases vinblastine sensitivity in drug-sensitive and drug-resistant cancer cells. *Cancer Res* 59: 880-885, 1999.
39. Selzner M, Bielawska A, Morse MA, Rüdiger HA, Sindram D, Hannun YA and Clavien PA: Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. *Cancer Res* 61: 1233-1240, 2001.
40. Bedi A, Zehnbauser BA, Barber JP, Sharkis SJ and Jones RJ: Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. *Blood* 83: 2038-2044, 1994.
41. Deming PB, Schafer ZT, Tashker JS, Potts MB, Deshmukh M and Kornbluth S: Bcr-Abl-mediated protection from apoptosis downstream of mitochondrial cytochrome c release. *Mol Cell Biol* 24: 10289-10299, 2004.
42. Bueno-da-Silva AE, Brumatti G, Russo FO, Green DR and Amarante-Mendes GP: Bcr-Abl-mediated resistance to apoptosis is independent of constant tyrosine-kinase activity. *Cell Death Differ* 10: 592-598, 2003.
43. Altieri DC: Survivin and IAP proteins in cell-death mechanisms. *Biochem J* 430: 199-205, 2010.
44. Kreuger MR, Grootjans S, Biavatti MW, Vandenabeele P and D'Herde K: Sesquiterpene lactones as drugs with multiple targets in cancer treatment: Focus on parthenolide. *Anticancer Drugs* 23: 883-896, 2012.
45. Saikali M, Ghantous A, Halawi R, Talhouk SN, Saliba NA and Darwiche N: Sesquiterpene lactones isolated from indigenous Middle Eastern plants inhibit tumor promoter-induced transformation of JB6 cells. *BMC Complement Altern Med* 12: 89, 2012.
46. Viennois E, Xiao B, Ayyadurai S, Wang L, Wang PG, Zhang Q, Chen Y and Merlin D: Micheliolide, a new sesquiterpene lactone that inhibits intestinal inflammation and colitis-associated cancer. *Lab Invest* 94: 950-965, 2014.
47. Yeh CT, Huang WC, Rao YK, Ye M, Lee WH, Wang LS, Tzeng DT, Wu CH, Shieh YS, Huang CY, *et al*: A sesquiterpene lactone antrocin from *Antrodia camphorata* negatively modulates JAK2/STAT3 signaling via microRNA let-7c and induces apoptosis in lung cancer cells. *Carcinogenesis* 34: 2918-2928, 2013.