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Vobtusine from *Voacanga foetida* (Blume) Rolfe Induces Apoptosis *via* Activation of Caspase Pathway in Human HL-60 Leukemia Cell Line

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Abstract*— Vobtusine is an aspidosperma-aspidosperma alkaloid isolated from alkaloid DCM base fraction of the bark of *Voacanga foetida* (Blume) Rolfe (Apocynaceae). In this study, the effect of vobtusine on the cell cycle, apoptosis induction, and Bcl-2 family protein expression were investigated by flow cytometr, DNA fragmentation analysis, and western blotting, respectively. The results of cell cycle analysis indicated the ratio of the number of cells in each phase did not have significant differences depend on vobtusine concentration although cell number in G_1 phase had tendency to decrease according to the increasing of vobtusine concentration. Besides, the sub-G_1 phase population of HL-60 cells treated with vobtusine was increased compared with that of cells without treatments (5.9%-23.8%). DNA fragmentation was observed from 20 µM, and the degree of fragmentation was dependent on vobtusine concentration. Caspase-3 activity increased 4.6 times compared to control. After being treated with caspase-9 inhibitor, vobtusine-induced elevation of caspase-3 activity decreased. This shows that caspase-3 activity depends on caspase-9. Vobtusine was also induced apoptotic cell death involved a mitochondrial by Bid activation and Bcl-xL downregulation. Therefore, vobtsusine-induced apoptosis process was initiated by caspase-9 *via* change of Bcl-2 family protein expression and executed by caspase-3, followed by cell death due to their proteolytic activity. These results indicated the mechanism action of vobtusine as anti-cancer compound *via* intrinsic pathway.

***Keywords*— vobtusine; cell cycle, apoptosis; caspases (-8, -9, -3); Bid; Bcl-xL.**

I. INTRODUCTION

Voacanga foetida (Blume) Rolfe (Apocynaceae), locally in West Sumatra known as"Tampa badak," is a small tree up to 20 meters high, with white latex; trunk diameter of up to 40 centimeters in diameter widely spread in Indonesia, Malaysia, and the Philippine. The barks and bark of *V. foetida* are commonly using to treat skin conditions such as itches, wounds, and swellings. The plant's latex has used externally to treat skin disorders [1]. The genus *Voacanga* is one source of indole alkaloids such as ibogaine, voacangine, and voacristine. This genus is also known to be a source of bisindole alkaloids or dimeric alkaloids. Bisindole alkaloids comprise two indole alkaloid monomers units joined by C-C, C-O-C, or C-N bonds [2],[3]. The plant contained high concentrations of alkaloids [4]. In continuation of our study on Sumatran plants [5-12]. Our further study was carried out on the barks of *V. foetida* since it has cytotoxicity activity [13]. We conducted the isolation work to get more compound for cell cycle, apoptosis [14-16], and protein expression [17]. Cell cycle can be defined as an ordered set of events culminating in cell growth and division into two

identical daughter cells. In normal cells, this complex process is initiated only in the presence of a mitogenic stimulus. Various proteins such as the cyclin dependent kinases; cyclins and cyclin dependent kinase inhibitors regulate the cell cycle. However, in cancer cells, this regulation of the cell cycle is lost and the cells continue to divide irrespective of the presence or absence of a mitogenic stimulus [18].

Apoptosis is the programmed cell death, which involves several proteins that play an essential role in the mechanism of cell death. The apoptosis triggered either an extrinsic apoptotic pathway or an intrinsic pathway. The key to the regulation and execution of intrinsic pathway is the Bcl-2 family of protein, which includes both pro-apoptosis (Bax, Bad, Bid) and pro-survival functions (Bcl-2, Bcl-xL, Mcl-1) [19].

The intrinsic pathway (mitochondria) occurs due to increased mitochondrial membrane permeability due to the influence of genetic damage, hypoxia, increased systolic Ca^{2+}, and oxidative stress. That triggers the binding of Bax pro-apoptotic protein with the outer mitochondrial membrane. This bond opens channels (pore) in the mitochondrial membrane so that cytochromes-c out of the mitochondria into the cytosol, cytochrome-c will bind with Apaf-1 to form apoptosomes and activate pro-caspase-9, then caspase-9 becomes active, caspase-9 will activate caspase-3, where caspase-3 is an enzyme essential to initiate apoptosis [20].

This study focuses on analyzing the mechanism action of vobtusine as an anti-cancer by testing the cell cycle, apoptosis, pro-apoptotic protein, and pro-survival protein expression, caspase-3, 8, and 9 expressions, as well as testing caspase-3 activity.

II. Material and Methods

A. General Experimental Procedures

Silica gel (Merck 7734), amino silica, and HP-20 were used for column chromatography (CC). Silica gel 60 F_{254} TLC Plates were used to monitor separations. The spots were imagined under ultraviolet lights at 254 and 366 nm. UV (in $CHCl_3$) and IR (KBr) spectra were taken on a Shimadzu UVmini-1240 and a Thermo FTIR spectrophotometer, respectively. EI-MS was found on a JEOL J MS HX-110A spectrometer. ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) were found on a Bruker ARX-500 ADV spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively. ^{1}H-^{1}H COSY, HMBC, and HMQC techniques used to analyze the spectra.

Cell culture monitored by the camera of ICX285, cold CCD F 0.95 for detection of luminescence, and device FAC-Scan used to measure the Sub-G_1 area. ECL Advance Western Detection System (GE Healthcare, Japan) was used for immune-complex detection. Glo-Max (Promega, Japan) was used to detect the activation of caspase-3. Cell culture was grown in a incubator at 37 °C and 5% CO_2. β-actin (1:4000, SIGMA, Japan) used as an internal standard. Primary antibodies were caspase-8, caspase-9, and caspase-3 (1:2000, BD Pharmingen, Japan); secondary antibodies were anti-rabbit IgG and anti-mouse IgG (1:2000, BD Healthcare, Japan); cell line HL-60 (human promyelocytic leukemia). Medium: RPMI-1640 (Wako, Japan) + 10% FBS (Fetal Bovine Serum, Bio-west, Japan) + 200 unit/mL penicillin / 200 μg/mL streptomycin (Wako, Japan), Trypsin-EDTA (0.25% Trypsin-EDTA, Gibco, Japan) was used for cell detachment. Trypan Blue 0.4% (Invitrogen, Japan) was performed under a microscope using a blood cell counter.

B. Plant Material

V. foetida plant was collected at the Biological Education and Research Forest Universitas Andalas, Padang, West Sumatra, Indonesia, in January 2019. The voucher specimen DR-167 identified by Dr. Nurainas and held at herbarium Universitas Andalas .

C. Extraction of V. foetida Bark

The dried air of the bark of *V. foetida* (5.2 kg) was grinded and extracted for five days with methanol (10 L). This maceration was repeated two times, extracts were evaporated under vacuum to *ca* 500 mL and next diluted with tartaric acid (3%, 500 mL) then partitioned with dichloromethane (5 × 500 mL). The acidic part was basified with sodium bicarbonate and then fractionated with dichloromethane (5 × 500 mL). The dichloromethane extract was evaporated under vacuum to give a crude alkaloid fraction as a dark brown gum (11.33 g)

A portion of DMC Base alkaloid fraction (10 g) were separated using column chromatography on amino silica as stationary phase and increasing amount of ethyl acetate in hexane as eluent. Fraction with contain similar pattern on TLC were combined and subjected to HPLC method using ODS MS-II column, 20 × 30 mm, eluent 3% ACN in H_2O (0.1% formic acid), the sample concentration used was 20 mg / mL, R = 0.16, Flow rate = 8 mL / min and injection volume of 10 mL. Similar fractions were combined, evaporated to give vobtusine (**1**) (Fig.1) (38 mg) as a yellowish amorphous from methanol which decomposed at 300 °C.

The UV absorption bands at λ_{max} /nm (MeOH) 239 nm (log ε = 4.09), 286 (log ε = 4.10) and 323 (log ε= 4.25). The IR spectrum showed ν_{max}/cm^{-1} (KBr) 3334, 2934, 1688. The EIMS: $[M]^+$ *m/z* 718 (95%), 700 (65), 562 (35), 504 (100), 138 (73), 110 (27). ^{1}H NMR (500 MHz/$CDCl_3$) δ 8.90 (1H, brs), 6.62-7.27 (7H, m), 7.17 (1H, brd, *J* 7.68 Hz), 6.80 (1H, brd, 7.68 Hz), 6.88 (1H, brt), 3.73 (3H, s), 3.79 (3H, s). ^{13}C NMR (125 MHz/$CDCl_3$) δ 167.3 (C-2), 54.2 (C-3), 51.3 (C-5), 45.3 (C-6), 55.3 (C-7), 138.0 (C-8), 121.8 (C-9), 121.0 (C-10), 128.1 (C-11), 109.6 (C-12), 143.4 (C-13), 40.0 (C-14), 87.8 (C-15), 94.7 (C-16), 27.7 (C-17), 64.7 (C-18), 35.1 (C-19), 48.0 (C-20), 69.3 (C-21), 169.0 (C=O), 51.3 (OMe), 93.8 (C-2'), 49.2 (C-3'), 52.2 (C-5'), 31.2 (C-6'), 56.3 (C-7'), 134.8 (C-8'), 115.1 (C-9'), 118.8 (C-10'), 111.2 (C-11'), 145.5 (C-12'), 137.5 (C-13'), 26.3 (C-14'), 80.8 (C-15'), 31.6 (C-16'), 32.7 (C-17'), 65.6 (C-18'), 37.0 (C-19'), 44.3 (C-20'), 64.4 (C-21'), 34.3 (C-22'), 46.6 (C-23'), 55.4 (OMe).

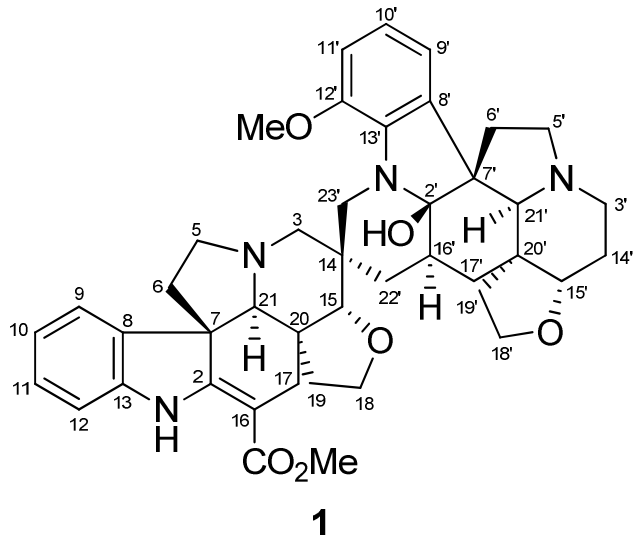


Fig. 1 Structure of vobtusine

D. Cytotoxic Activity

HL-60 (human blood premyelocytic leukemia) was seeded into 96-well microtiter plates at 10^4 cells per well. Cells were pre-incubated for 24 hours at 37 °C in a moisturized climate of 5% CO_2. Various compounds (10 μL) were added to the cell cultures then incubated at 37 °C for 48 hours. On the third day, 15 μL MTT solution (5 mg/mL) each cultured medium. After 2 hours of incubation, 100 μL of 10% SDS dissolved in 0.04 mol/L of HCl solution was added into each well to lyse the cells and solubilize the formazan crystals. The micropipette reader (Bio-Rad) is used to measure the optical density. We do three replicates of wells prepared for each sample. The difference in the absorbance between samples and controls is used to determine the living cells' ratio. The cytotoxic activity uses to indicated an IC_{50} value. Bisindole alkaloid vincristine and vinblastine are used as a positive control. The IC_{50} values against HL-60 cells were 0.87 and 1.6 nM, respectively.

E. Cell Cycle Analysis by Flow Cytometry

HL-60 cells (1×10^5 cells /6 cm dish) were incubated at 37 °C, 5% CO_2 for 24 hours. Vobtusine at concentrations of 40, 30, 20, and 10 μM were used to treat the cell. The negative control using DMSO, cell-treated with vobtusine centrifuged (1000 rpm, 10 minutes), then pellet washed using PBS, put to a 1.5 mL Eppendorf tube. 70% ethanol (1 mL) was added to cells and fixed overnight at -20 °C. Centrifuged (3000 rpm, 5 minutes at 4 °C), washed using PBS, added RNase (37 °C, 30 minutes, 100 μg/mL), centrifuged (3000 rpm, 5 minutes at 4 °C), washed using PBS. Added 500 μL PBS (0.2% Triton-X100), 50 μL propidium iodide (PI), and suspended at room temperature, the cell passes through the nylon mesh (30 μm), measuring the area associated with cell cycle capture, namely the Sub-G_1 area using FAC-Scan.

F. Apoptosis Analysis by DNA Fragmentation Analysis with DNA Ladder Method

10 mM EDTA-2Na, 0.5% SLS, 50 μL of 10% SDS and 50 mM Tris-HCl (pH 7.8) were added to cells and collected for lysis, 50 μL 10 mg/mL proteinase K was added, heated at 50 °C for 90 minutes. 50 μL 10 mg/mL RNase-A was added, heated at 50 °C for 30 minutes. 10 mg/mL glycogen, 0.6 mL sodium iodide solution, 6 M NaI, 13 mM ETDA-2Na, 0.5% SLS, and 26 mM Tris-HCl (pH 8.0) are warmed at 60 °C for 15 minutes. 1 mL of isopropanol was added, set aside for overnight at 4 °C, centrifuged 13.000 rpm, 15 minute, 4 °C). The precipitate was washed with 50% isopropanol and 70% EtOH, dissolved in a lysis buffer (DNA solution), separated with 2% agarose-TBE gel, and marked with ethidium bromide.

G. Western Blotting Analysis

1) Preparation of cell lysate: HL-60 cells treated with vobtusine, centrifuged (1000 rpm, 10 minutes), washed using PBS. Transferred to Eppendorf tube (1.5 mL), added to 0.5% sodium deoxycholate, 100 μL lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM orthovanadate sodium, 0.1 mM NaF), 0.5% Triton X-100, homogeneous with a 22 G needle, centrifuged (13,000 rpm, 20 minutes, 4 °C), and obtained a supernatant for SDS-PAGE.

2) Transfer of protein and staining: The resulting cells lysate was separated using 12.5% polyacrylamide running gel, applied to a gel, separated with a stacking gel (10 V), and a running gel (20 V). After separation, the polyvinylidene difluoride (PVDF) membrane was performed by a semi-dry method. Separate gels with buffer A (25 mM Tris, MeOH 5%), buffer B (25 mM Tris, 40 mM 6-aminohexanoic acid, MeOH 5%), and buffer C (0.3 M Tris, MeOH 5%), soaked in filter paper. The PVDF membrane was sandwiched between them and energized for 45 minutes at a voltage of 15 V.

3) Primary and secondary antibody treatment: PVDF membrane transfer to TBS/T buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20] containing 0.5% skim milk. Blocking treatment at 37 °C, 60 minutes. The blots were washed with TBS/T and then transferred to the primary antibody treatment, diluted with TBS/T with 5% skim milk at 37 °C, 60 minutes, washed with TBS/T, and then transferred to secondary antibody treatment, diluted with TBS/T and then transferred to immune-complex detection.

H. The Influences of Caspase-8 Inhibitor and Caspase-9 Inhibitor on Caspase-3 Activity

HL-60 cells (1×10^4 cells/well) in 96-well plate were incubated (37 °C, 5% CO_2) for 24 hours. Vobtusine (20 and 10 μM) dissolved in DMSO, respective 20 μM of caspase-8 inhibitor and caspase-9 inhibitor (BioVision) were co-added. Only DMSO was added to the negative control. After acting for 24 hours, then detect the activation of the caspase-3 index. The activation rate of caspase-3 was represented as the relative value as DMSO-treated sample is 1.0.

III. Results and Discussions

The characterization of the isolated compound was carried out by the spectroscopic method. The UV absorption bands at max 239 nm ($\log \varepsilon$ =4.09), 286 nm ($\log \varepsilon$ =4.10) and 323 nm ($\log \varepsilon$ =4.25) indicated two indole chromophores. The IR spectrum KBr disk showed ν = 3334 cm^{-1} for NH and OH, ν

= 2934 cm^{-1} CH aliphatic and ν = 1688 cm^{-1} for C=O. The EIMS showed $[M]^+$ peak at *m/z* 718, analyzing for $C_{43}H_{50}N_4O_6$. The *m/z* 700 showed a loss of H_2O followed by retro Diels-Alder reaction to produce *m/z* 562 $[C_{35}H_{36}N_3O_4]^+$ (Fig. 2).

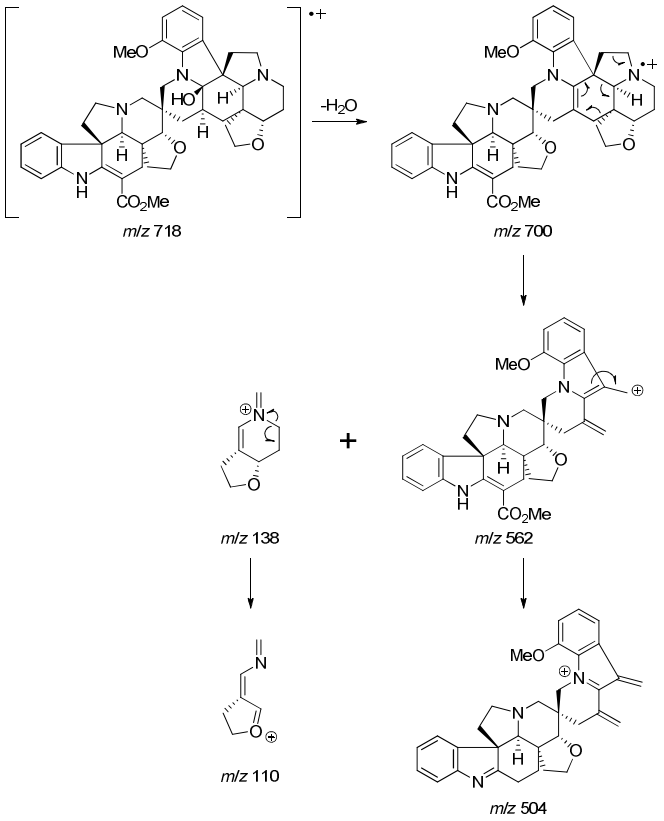
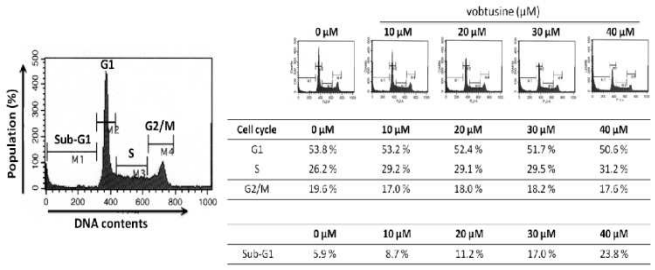


Fig. 2 Fragmentation of vobtusine (**1**)

The ^1H NMR spectrum in $CDCl_3$ indicated NH (δ_H 8.90) and multiplet at δ_H 6.6-7.3 ppm for 7 aromatic protons. The signal at δ_H 7.17 (1H, brd, 7.68 Hz) was assigned as H-11', and at δ_H 6.80 (brd, 7.68 Hz) was designated as H-9'. The signal at δ_H 6.88 (2H, brt) was assigned as H-10. The ^{13}C spectrum showed a similar chemical shift with vobtusine (**1**).

We evaluated for vobtusine cytotoxicity against HL-60 cell line. The cytotxic activity show that vobtusine have a moderate cytotoxic activity with IC_{50} 15.8 µM. The effect of vobtusine on the cell cycle was determined by the distribution of vobtusine-treated cells in the G_1, S, and G_2 / M phases detected by flow cytometry [21],[22]. The results of cell cycle analysis indicate the ratio of the number of cells in each cell cycle did not have significant differences depend on vobtusine concentration although cell number in G_1 phase have tendency to decrease according to the increasing of vobtusine (Fig. 3). Besides, the sub-G_1 phase population of cells treated with vobtusine was increased compared with that of cells without treatments. These results suggested that vobtusine induced apoptosis in HL-60 cells, which is initiated from G_1 phase.



Cell cycle	0 µM	10 µM	20 µM	30 µM	40 µM
G1	53.8 %	53.2 %	52.4 %	51.7 %	50.6 %
S	26.2 %	29.2 %	29.1 %	29.5 %	31.2 %
G2/M	19.6 %	17.0 %	18.0 %	18.2 %	17.6 %

	0 µM	10 µM	20 µM	30 µM	40 µM
Sub-G1	5.9 %	8.7 %	11.2 %	17.0 %	23.8 %

Fig. 3 The effects of vobtusine on the cell cycle of HL-60. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours, and added vobtusine/DMSO (concentrations of 40, 30, 20, and 10 µM). The negative control only added DMSO on HL-60 cells. Vobtusine is no concentration-dependent of G_1, S, and G_2/M phases whereas concentration-dependent of sub G_1 phase. The percentage of sub G_1 cells increased from 5.9% at 0 µM to 23.8% at 40 µM.

One of the apoptotic induction processes is DNA fragmentation in the nucleus, by examining DNA fragmentation from HL-60 cell lines treated with vobtusine. Showed that the occurrence of DNA fragmentation occurred at vobtusine concentrations of 20 µM or more. DNA fragmentation usually occurs during the final stages of the apoptotic process. These results indicate that vobtusine induces apoptosis, as evidenced by the presence of DNA fragmentation in HL-60 cell lines during treatment with vobtusine (Fig. 4).

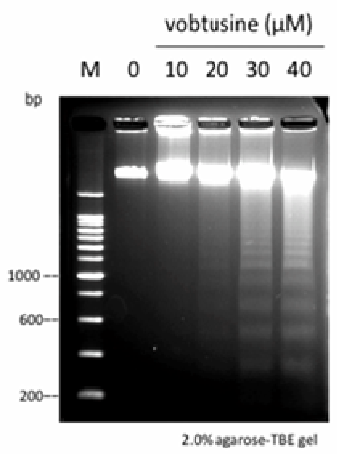


Fig. 4 Detection of apoptotic DNA ladder in vobtusine-treated HL-60 cells. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours, and added vobtusine/DMSO (concentrations of 40, 30, 20, and 10 µM) for 24 hours. Genomic DNA was extracted from HL-60 cells, which was separated by 2% agarose gel electrophoresis. Lane 1; marker size 200-bp, lane 2; DMSO as the negative control, lanes 3-6; vobtusine in various concentrations.

Apoptosis is mainly due to the continuous cell division and activation of the protein caspase, a cysteine protease directed by aspartate. Generally, caspase occurs in cells in the form of inactive pro-caspase, and when activated, it can trigger other pro-caspases, allowing the protease cascade's initiation. To confirm whether vobtusine can activate cascade of caspases that correlate with the enzymatic activity of caspase-3, -8, and -9, we investigated on HL-60 cells by observing at the expression of pro-caspase-3, -8, and -9 (Fig. 5). These detection by WB revealed that the three caspase precursors decreased depend on vobtusine concentration.

Furthermore, cleaved caspase-3 expression increased depend on vobtusine concentrations. These results indicate that the mechanism of action of vobtusine in inducing apoptosis by activating caspase-3.

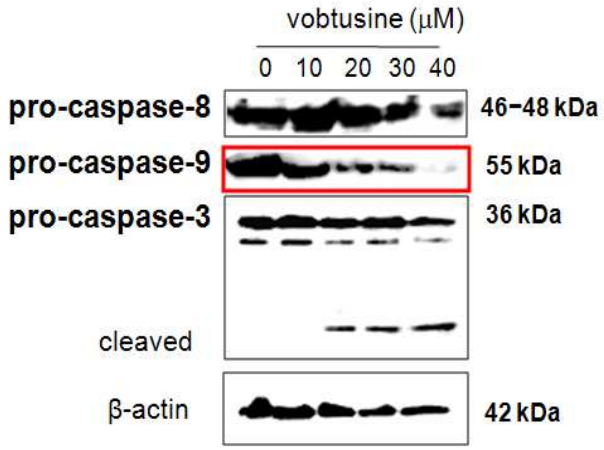


Fig. 5 Protein expression of pro-caspase-8, pro-caspase-9, and pro-caspase-3/caspase-3 in HL-60 cells treated with vobtusine at concentrations 0, 10, 20, 30, and 40 μM. HL-60 cells (1×10^5 cells/6 cm dish) incubated for 24 hours with vobtusine (10 - 40 μM). The resulting cell lysate was separated using SDS-PAGE (12.5%), and transferred to PVDF membrane. Dose-dependent decrease of pro-caspase-8, pro-caspase-9 and pro-caspase-3 was detected. Increase of cleaved caspase-3 were also detected in vobtusine-treated cell lysate (20-40 μM).

Vobtusine can induce apoptosis through caspase-3 activation (Fig. 6). When vobtusine combined with caspase-9 inhibitors, caspase-3 activation suppressed, this means that caspase-3 activity depends on caspase-9 activation. On the other hand, when vobtusine is combined with caspase-8 inhibitor slight decrease was observed. This shows that vobtusine activity in inducing apoptosis through caspase-3 activation is mainly depend on caspase-9 activation, not too dependent on caspase-8 activation. Because of the mechanism of vobtusine in inducing apoptosis through activation of caspase-9 without or a little activation of caspase-8, it suggested that the mechanism of induction apoptosis with vobtusine is through the intrinsic pathway and not through extrinsic pathway.

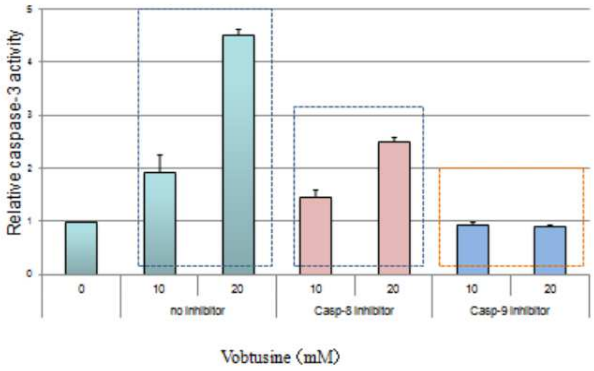


Fig 6 Effects of caspase-8 and caspase-9 inhibitor against caspase-3 activation. HL-60 cells (1×10^4 cells/well) in 96-well plate were incubated for 24 hours. Vobtusine (20 and 10 μM) dissolved in DMSO, respective 20 μM of caspase-8 inhibitor and caspase-9 inhibitor were co-added. Only DMSO was added to the negative control. The activation rate of caspase-3 was represented as the relative value as DMSO-treated sample is 1.0.

The mechanism of action of vobtusine in inducing apoptosis is through the intrinsic pathway involves the performance of the Bcl-2 family protein, namely pro-apoptotic proteins (Bid, Bax, Bad) and pro-survival proteins (Bcl-2, Mcl-1, Bcl-xL). To find out whether vobtusine works by increasing pro-apoptotic proteins expression or inhibiting pro-survival protein expression, an analysis of pro-apoptotic proteins expression and pro-survival protein was analyzed by western blotting. The results of this study indicated that the expression of Bid and Bcl-xL protein in vobtusine-treated HL-60 would reduce (Figure 5). Cleaved t-Bid increasing, is reported to activate Bax and Bak, is expected by the downregulation of Bid. Therefore, vobtusine is speculated to induce apoptosis via caspase-9 and regulated by upstreamed activities of Bcl-xL and Bid (Fig. 7).

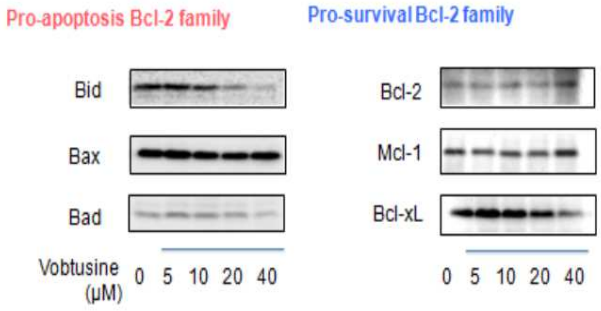


Fig. 7 Protein expression of Bcl-2 family in HL-60 cells treated with vobtusine. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours with vobtusine (5 - 40 μM). The resulting cell lysate was separated using SDS-PAGE (12.5%), and transferred to PVDF membrane. Dose dependent decrease of Bid and Bcl-xL were detected.

IV. Conclusions

The present study concluded that the mechanism action of vobtusine as anticancer drugs by inducing apoptotic cell death, through the mitochondrial pathway, including caspase-9, caspase-3, Bid activation, and Bcl-xL downregulation on HL-60 cell line.

Acknowledgment

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