Corchorusin-D Directed Apoptosis of K562 Cells Occurs through Activation of Mitochondrial and Death Receptor Pathways and Suppression of AKT/PKB Pathway

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Key Words
Corchorus acutangulus • Corchorusin-D • Saikosaponin • K562 • Death receptor

Abstract
Saponins, plant glycosides, have been reported to possess anti-cancer properties. Therefore the effect of corchorusin-D (COR-D), a compound isolated from Corchorus acutangulus, was studied in the chronic myelogenous leukemic cell line K562, using MTT assay, phase contrast and confocal microscopy, annexin V binding, cell cycle analysis and western blotting. COR-D inhibited cell growth in K562 cells and showed increased number of Annexin V FITC binding cells. Characteristic apoptotic changes, seen under phase contrast and confocal microscopes with accumulation of cells in the sub-G0 phase. The apoptosis involved drop in Bcl-2/Bax ratio, loss of mitochondrial membrane potential, release of cytochrome c in cytosol followed by activation of caspases 9 and 3, and cleavage of PARP. Down-regulation of pro-caspase 10 was observed along with formation of death-inducing signaling complex between TNF-R1 and TRADD. COR-D suppressed PDK1 and AKT with activation of MAP kinase family members ERK1/2, JNK1/2 and p38. Thus it induced apoptosis by activating mitochondrial and death receptor pathways and suppressing AKT/PKB rather than MAP kinase pathway. Significant enhancement of apoptosis, noted using specific inhibitors of ERK1/2, p38 and JNK1/2, suggests that COR-D can enhance apoptosis in K562 cells in combination with MAP kinase inhibitors.
Introduction

Uncontrolled cell proliferation that results from disrupted cell death signaling is a critical component of many research areas, especially cancer biology [1]. In human chronic myelogenous leukemia (CML), Bcr-Abl fusion gene encoded cytoplasmic protein p210 bcr/abl exhibited constitutive tyrosine kinase activity and initiated signaling through multiple pathways [1]. This p210 bcr/abl-initiated signaling decreases the ability of a variety of stimuli to induce apoptosis in vitro [2].

Apoptosis, a major form of cell death, is characterized by a series of stereotypic morphological features [3]. In general, two major pathways of apoptosis, intrinsic and extrinsic, have been described [4]. In the intrinsic pathway diverse pro-apoptotic signals stimulate the translocation of cytochrome c from mitochondria to cytosol that promotes caspase 9 and caspase 3 activation. This in turn cleaves PARP which has a role in DNA damage induced apoptosis [5, 6]. In the extrinsic pathway, apoptosis is mediated by death receptors such as Fas or tumor necrosis factor receptors [7] and involves caspase 8/10 activation [8].

AKT, also known as protein kinase B (PKB), is a serine/threonine protein kinase that plays a key role in apoptosis [9]. Activated PKB/AKT provides a survival signal that protects cells from apoptosis induced by various stresses [10]. Mitogen-activated protein kinases (MAPKs), another group of the serine/threonine family of kinases, phosphorylate upon stimulation and such phosphorylation events can modulate gene expression, mitosis, proliferation, motility and apoptosis [11-13]. Saikosaponins and saikosaponin-like compounds have been reported to possess potent anti-cancer activity [14-18]. Since Corchorusin-D (COR-D), isolated from Corchorus acutangulus, is a saikosaponin like compound, earlier we investigated its anti-leukemic activity in U937 and HL-60 cell lines and found that COR-D induced apoptosis in these cells [19]. In the present study an attempt was made to investigate the apoptotic effect of COR-D on K562 cells, a chronic myelogenous leukemic cell, and to elucidate the signaling pathways involved in apoptosis induced by COR-D in this cell line.

Materials and Methods

Materials and chemicals

RPMI 1640 medium, fetal bovine serum (FBS), HEPES, streptomycin, penicillin, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DAPI (4′,6-diamidino-2-phenylindole), 7-AAD (7-Aminoactinomycin D), JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide, Sigma), propidium iodide (PI), Annexin V-FITC (sigma) and general reagents were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bcl-2, Bax, Bcl-xl, PARP, Bid, pro-caspase 3, pro-caspase 8, caspase 9, pro-caspase 10, cytochrome c, TNF-R1, TNF-R2, TRADD, Beta-actin, PDK1, p-PDK1, AKT, p-AKT (ser-473 and Thr-450), MEK, p-MEK, ERK, p-ERK, p-38, pp-38, JNK1/2, p-JNK1/2 were purchased from Cell Signaling (USA). Inhibitors for ERK (U0126) and for JNK1/2 (SP600125) and p38 (SB203580) were purchased from Sigma and Calbiochem respectively. All other chemicals and solvents were of high purity grade and purchased from local firms.

Cell culture

Human leukemic cell line K562 was purchased from National Facility of Animal Tissue and Cell Culture, Pune, India. Cells were cultured in RPMI 1640 medium supplemented with 10 % heat inactivated FBS (GBCO BRL, USA) and antibodies in appropriate conditions. In all the experiments untreated leukemic cells were used as control.

Cell viability assay

Cells (1 × 10^6) were treated with different concentrations of COR-D (25-150 µM). Control cells were then supplemented with complete media. Following treatment, cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] assay [20] and the pictorial views of cells were captured by phase contrast microscopy. For the detection of cell viability, cells (1×10^6) were
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Cultured and treated with IC\textsubscript{50} concentration of COR-D for 24 h, then processed and stained with 7-AAD. After incubation in dark for 15 min, these were subjected to flow-cytometry. For each sample 1×10\textsuperscript{4} cells were acquired and the data were analyzed by Cell Quest Pro software (BD FACS Calibur) [21].

**Confocal microscopy**

After treatment with COR-D at the IC\textsubscript{50} concentration, the cells were harvested, washed with phosphate buffer saline (PBS), and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. The fixed cells were washed with PBS, stained with 2.5 µg/ml of DAPI (4',6-diamidino-2-phenylindole) solution for 10 min at room temperature, washed twice with PBS, and analyzed using a confocal laser-scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany) with excitation/emission wavelengths of 358/461 nm.

**Detection of exposed surface phosphatidylserine (PS) by flow-cytometry**

Cells (1×10\textsuperscript{6}) were exposed to COR-D (IC\textsubscript{50}) for 24 h. Evaluation of apoptosis was performed using annexin V-FITC and propidium iodide (5 mg/ml) according to the manufacturer's instructions. At least 1×10\textsuperscript{4} cells were examined and the data were analyzed by CellQuest Pro software (BD FACS Calibur) [21].

**Flow-cytometric detection of cell cycle phase distribution**

Cells (1×10\textsuperscript{6}) were treated with COR-D at IC\textsubscript{50} concentration for 24 h, harvested, washed and fixed overnight in ethanol. The fixed cells were washed twice in PBS and treated with RNase in 38 mM sodium citrate buffer for 1 h in darkness [22]. Cell cycle phase distribution of nuclear DNA was determined on FACS. At least 1×10\textsuperscript{4} cells were examined and the data were analyzed by CellQuest Pro software (BD FACS Calibur). A histogram of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) was displayed.

**Mitochondrial membrane potential (MMP) assay**

Alteration in mitochondrial membrane potential was analyzed by flow-cytometry using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, Sigma). K562 cells (1×10\textsuperscript{6}/ml) were treated with different concentrations (60-100 µM) of COR-D for 24 h. The cells were harvested, washed, re-suspended in PBS and incubated with JC-1 (25 µM) at 37°C for 15 min. These were then washed with PBS and 1×10\textsuperscript{4} cells were acquired per sample for analysis by BD Cell Quest Pro software. JC-1 monomer emits at 530 nm (FL-1 channel) and aggregates emit at 590 nm (FL-2 channel) [23].

**Immunoblotting and immunoprecipitation**

K562 cells were treated with different concentrations (60-100 µM) of COR-D for 24 h. After cell lysate preparation, an equivalent amount of protein (40 µg) from each sample was resolved by SDS-PAGE (10%) and electro-transferred into PVDF membrane. The membrane was blocked by dipping in 3% TBST-BSA (TBS containing 0.05% Tween 20), incubated with appropriate primary antibody overnight at 4°C, washed with PBS containing 0.1% Tween 20, and incubated with the appropriate HRP-conjugated secondary antibody. The immune complexes were detected by Super Signal West Pico Chemiluminescent substrate kit (Pierce).

In the immunoprecipitation assay, whole cell lysate (300 µg) was incubated with specific antibody (1:100 dilution) and incubated overnight at 4°C. The immunocomplex was precipitated by protein A sepharose 4B, the resultant complex was washed and resolved in non-reducing SDS PAGE, and western blot was carried out.

**Sub cellular fractionation**

Following specific treatment, cytosolic and pellet (mitochondrial) fractions were generated using a reagent-based sub-cellular fractionation technique according to the manufacturer’s protocol (Pierce). Briefly, 1×10\textsuperscript{7} cells were harvested by centrifugation at 800 g, washed in PBS, and re-pelleted. Reagent A (400 µl) was added to the cells, vortexed at medium speed, and incubated on ice for 2 min. Then 5 µl of reagent B was added and incubated on ice for 5 min. This was followed by the addition of 400 µl of reagent C and proper mixing. Following centrifugation at 800 g and 4°C for 10 min, the supernatant containing cytoplasmic protein was separated from the pellet comprising mitochondria and cellular debris and further purified by centrifugation at 13,000 g and 4°C for 10 min. Then 400 µl of mitochondrial isolation reagent was added to the pellet and centrifuged at 12,000 g for 5 min. The supernatant was discarded and the...
mitochondrial pellet was maintained on ice before downstream processing. Cytochrome c and proteins of Bcl-2 family were detected by Western blot analysis.

**Statistical analysis**

All the experiments were performed three times. Data are expressed as the mean ± standard error of the mean (SEM). A significant difference from the respective controls for each experimental test condition was assessed using Student’s t test for each paired experiment. A value of p < 0.01 was regarded as indicating statistical significance. Densitometry was performed using Image J analysis software (NIH).

**Results**

**COR-D inhibits cell growth and induced apoptosis in K562 cells**

COR-D inhibited proliferation of K562 cells in concentration and time-dependent manner (Fig. 1A); this was further supported by 7-AAD positivity (Fig. 1B). IC_{50} values were 81.25±0.592 µM (24 h), 70.95±2.045 µM (48 h) and 60.373±1.688 µM (72 h). Phase
contrast and confocal microscopy showed morphological changes like cell shrinkage, nuclear condensation and chromosomal fragmentation in COR-D treated cells (Fig. 1C). The treatment induced phosphatidylserine (PS) externalization in K562 cells after 24 h, which is a hallmark of apoptosis (Fig. 1D). Cells treated for 24 h with COR-D at IC\textsubscript{50} showed a significant increase in percentage of cells in sub-G0 phase (Fig. 1E). These results together suggested that COR-D induced apoptosis in K562 cells.

**Involvement of mitochondria and mitochondrial anti- and pro-apoptotic proteins in COR-D induced K562 cell death**

As COR-D treatment appeared to shift the cells towards apoptosis, an attempt was made to confirm the involvement of mitochondria through JC-1 staining. Accordingly, COR-D treated K562 cells were subjected to FACS analysis using JC-1, which showed mitochondrial membrane depolarization in a concentration-dependent manner (Fig. 2). This suggested that the mitochondrial membrane potential was disrupted, resulting in cytosolic accumulation of monomeric JC-1. To reveal the mitochondrial pathway of apoptosis, the expression levels of anti- and pro-apoptotic proteins Bcl-2, Bcl-xL and Bax were studied by Western blot analysis. A concentration-dependent decrease in Bcl-2 and Bcl-xL and increase in Bax were observed in the total cell lysate (Fig. 3A, 3B). In order to analyze the mechanistic role of Bax and cytochrome c in mitochondria mediated apoptosis, cytosol and mitochondria were fractionated and the expressions of these proteins were studied. Cytochrome c showed a concentration-dependent increase in cytosol and decrease in mitochondria (Fig. 3C); as expected Bax showed a gradual decrease in cytosol and increase in mitochondria (Fig. 3C). Evaluation of the role of caspases in mitochondrial pathway thereafter showed that caspase 9 and caspase 3 were activated after COR-D treatment (Fig. 3D). After 24 h of treatment, the DNA repairing enzyme PARP was cleaved to an 85 kDa inactivated form (Fig. 3D). These

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**Fig. 2.** Mitochondrial membrane depolarization following exposure of K562 cells to COR-D. Cells were treated with different concentrations (60-100 µM) of COR-D for 24 h, stained with JC-1 for 15 min at 37°C, and analyzed in FACS to determine the shift from J-aggregates to cytoplasmic monomers.
results confirmed that the cells undergo apoptosis through the mitochondria mediated pathway after treatment.

Fig. 3. Expression profile of mitochondrial pro- and anti-apoptotic proteins in K562 cells following COR-D treatment. Cell lysates were prepared after treating the cells with different concentrations (60-100 μM) of COR-D for 24 h. Untreated cells were used as control. Immunoblotting was performed as described in Methods. Beta actin was used as internal loading control. (A) Profiles of Bax, Bcl2 and Bcl-xl. (B) Graphical representation of fold change of Bax, Bcl-2 and Bcl-xl. (C) Cytosolic and mitochondrial fractions showing expression levels of Bax and cytochrome c. (D) Expression profiles of caspase 3, 9 and PARP in total cell lysate and densitometric analyses of (E) Cytosolic cytochrome c, (F) Cytosolic Bax, (G) Mitochondrial cytochrome c, (H) Mitochondrial Bax, (I) Caspase 9, (J) Pro-caspase 3, and (K) PARP, which indicate the fold change (in terms of %) of each protein compared with control. Typical results from three independent experiments are shown. *p < 0.01 implies significant difference in comparison to control group.
COR-D mediated activation of death receptor pathway

The efficacy of COR-D on death receptor pathway was then evaluated. The results proved that there was no significant change in activation of caspase 8 (Fig. 4A). As it is known that Bid is a direct substrate of activated caspase 8, expression level of Bid was examined in COR-D treated K562 cells. It was found that there was no significant change in Bid expression (Fig. 4A), ruling out caspase 8 mediated Bid cleavage. So, caspase 8/Bid pathway was not activated in K562 cells upon COR-D treatment. However, a concentration-dependent down-regulation of pro-caspase 10 was noticed (Fig. 4A) along with up-regulation of some TNF family members like TNF-R1 (up-regulated up to 70 µM), TNF-R2 and TRADD (up-regulated up to 80 µM) (Fig. 4B). This indicated involvement of the death receptor pathway in the apoptotic effect. In order to establish this, efforts were made to observe DISC (death-inducing signaling complex) formation between TNF-R1 and TRADD by immunoprecipitation (Fig. 4C). The association between TNF-R1 and TRADD was found to be maximum at 60 µM concentration of COR-D and decreased at concentrations above 70 µM. This suggested that at higher concentrations of COR-D, cells were in the process of undergoing apoptosis, destabilizing the complex. So it can be concluded that the compound induced apoptosis through the death receptor pathway also, along with the mitochondrial pathway.
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**COR-D induced apoptosis through suppression of AKT pathway**

In trying to find out the role of COR-D on AKT/PKB survival pathway in K562 cells, we noticed that COR-D suppressed the phosphorylation of AKT at ser-473 and thr-450 sites (Fig. 5). PDK1, the upstream kinase of AKT, was activated and showed a constant concentration-dependent down-regulation (Fig. 5). Therefore the compound appears to suppress the AKT/PKB pathway through PDK1 and induce apoptosis.

**COR-D induced MEK dependent ERK activation**

ERK (extracellular signal-regulated kinase) is a MAP kinase family member which is involved in a variety of functions like proliferation, differentiation and survival. Evaluation of the effectiveness of COR-D on ERK1/2 revealed that ERK was indeed activated (Fig. 6A). As MEK1/2 is the direct up-stream protein kinase of ERK, activation of MEK was checked. This revealed a constant concentration-dependent up-regulation of MEK (Fig. 6A), suggesting that activation of ERK was MEK dependent. To confirm this, MEK 1/2 was inhibited with 20 µM U0126 for 2 h resulting in total inhibition of ERK at 18 h and 24 h (Fig. 6B). Checking cell viability thereafter by MTT assay showed that cell death was enhanced after inhibiting MEK1/2 (Fig. 6C). Therefore, though ERK was not suppressed by COR-D, total inhibition occurred and cell death was enhanced when the directly upstream protein MEK was inhibited.
Fig. 6. Expression profiles of MAP kinase signaling proteins following COR-D treatment. Immunoblotting and flow-cytometry were determined as described in Methods. Total forms of respective proteins were used as loading controls. (A) Concentration-dependent expression pattern was observed for MEK, p-MEK, ERK1/2, p-ERK1/2, JNK1/2, p-JNK1/2, p38 and p-p38. (B) Expression level of ERK after inhibiting MEK. (C) Graphical representation of enhancement of apoptosis after inhibiting ERK. (D) Enhancement of COR-D induced apoptosis in K562 cells after inhibiting P38 (with SB203580) and JNK (with SP600125). The fold changes of the phosphorylated forms of (E) p-MEK, (F) p-ERK, (G) p-JNK and (H) p-p38 of COR-D treated cells were determined by densitometric analysis. The values indicate the fold change (in terms of %) of each protein compared with control. Typical results from three independent experiments are shown. *p < 0.01 implies significant difference in comparison to control group.
**COR-D induced activation of JNK1/2 and p38 kinase in K562 cells**

Western blot analysis was performed to investigate the role of stress related p38 and JNK1/2 kinases in K562 cells. This revealed that these kinases underwent a concentration-dependent activation (Fig. 6A). To confirm that the activation of the kinases is responsible for apoptosis or survival, p38 and JNK1/2 expressions were inhibited by SB203580 and SP600125. Cell death increased thereby, proving that COR-D in combination with JNK1/2 and p38 inhibitors must have enhanced cell death (Fig. 6D).

**Discussion**

Failure of apoptosis leads to an imbalance in cell number, which in turn leads to tumorigenesis. Hence induction of apoptosis has emerged as an important strategy for clinical cancer therapy [24]. Nowadays the development of herbal remedies for the treatment of cancer is emerging because of their minimal side effects [25, 26], which target different pathways [27, 28]. Significant annexin V-FITC positivity, accumulation of cells at sub-G0 phase, and typical morphological changes indicated that COR-D induced apoptosis in K562 cells. The observation led us to believe that the compound induces decrease in mitochondrial membrane potential, decrease in Bcl-2/Bax ratio, and release of cytochrome c from mitochondria to cytosol, facilitating caspase 9 activation; it also up-regulates the downstream pathways leading to caspase 3 activation and PARP cleavage. It can thus be concluded that apoptosis followed the mitochondria-dependent intrinsic pathway. Bid, a Bcl-2 family protein, is the substrate of activated caspase 8, whose activation is regulated by the death receptor, mainly through Fas/FasL interaction [29]. In the present case, there was no significant change in pro-caspase 8 and Bid expression; this is because caspase 8 activation is regulated mainly through the Fas/FasL interaction but K562 cells are Fas/FasL deficient [30]. The absence of caspase 8 activation prevents cleavage of bid in this type of cells. Although the highest concentration of COR-D induced lower level of Bid expression, this is likely to be due to cell death induced Bid degradation because at the highest concentration PARP was already cleaved. COR-D induces caspase 10 activation in K562 cells, which indicates that the death receptor pathway is activated by TNF receptor rather than Fas/FasL. From Fig. 4 it can be seen that the association between TNF-R1 and TRADD was maximum at low concentrations of COR-D; at higher concentrations, cells were undergoing apoptosis by destabilizing the complex. This demonstrated that the death receptor pathway was also activated.

The efficacy of COR-D over AKT/PKB pathway was then evaluated as it is known that activated PKB/AKT provides a survival signal to the cells and this protects apoptosis induced by various stresses [31]. The results showed that the compound activated the upstream kinase PDK1, which in turn suppressed AKT at ser-473 and Thr-450 sites. Thus it inhibits the AKT/PKB pathway to enhance apoptosis.

Investigations on the roles of the MAP kinase family members in regulating COR-D induced apoptosis showed that ERK and the upstream kinase MEK were up-regulated. The compound could not suppress ERK; but cell death was enhanced after inhibiting MEK, which suggested that ERK up-regulation in COR-D induced cell death is MEK dependent.

JNK and p38, the two kinases associated with different cellular processes including cell growth, differentiation, transformation and apoptosis [13, 14, 32], were activated in response to COR-D treatment. But cell death was enhanced when JNK and p38 were pre-inhibited with their specific inhibitors. This proved that the activation of p38 and JNK was associated with cell survival or cellular protection, as COR-D induced cell death was enhanced in pre-inhibited condition of p38 and JNK.

In conclusion, COR-D can induce apoptosis in K562 cells by activating intrinsic and extrinsic pathways and suppressing the AKT/PKB pathway. However, MAPK family members ERK, p38 and JNK were not involved in the apoptotic response. The results suggested that COR-D in combination with MAP kinase inhibitors can enhance apoptosis in K562 cells.
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