Apoptotic effect of COR-D on melanoma cells
INTRODUCTION

Cancer is a major public health problem in the world as brought out in the review included in chapter I. In recent years, there has been a substantial increase in studies on the effects of plant derived compounds in cancer prevention and treatment (Carvalho, 2010). Of all the types of cancer the present, the dissertation was tailored to provide novel therapies (in the form of natural products) to two specific types: leukemia and melanoma. More specifically, the triterpenoid saponin COR-D, isolated from Corchorus acutangulatus and structural analogue of saikosaponins many of which elicit anti-cancer effect, was selected for studying the effect on these two cancer types. The present chapter describes its efficacy in melanoma cases.

One of the main targets of cancer chemotherapy is to search for and develop new molecules and/or therapies, which can selectively induce apoptosis in cancer cells (Denicourt and Dowdy, 2004). Studies have reported that saikosaponin b2 inhibits the proliferation of melanoma cells (Zonga et al., 1996; Zong et al., 1998; Fujioka et al., 2003). Based on the structural similarity with saikosaponin D and COR-D, the pharmacological activity of COR-D has been investigated. Antiproliferative activity of and apoptosis induction by COR-D in melanoma B16F10 cells were studied. The results from MTT assay showed the anti-proliferative effects of COR-D on these cells. Moreover, the induced cell apoptosis was associated with decrease in the mitochondrial transmembrane potentials ($\Delta$ym) and Bcl-2/Bax ratio, together with the activation of caspase 9 and 3 and PARP degradation. Taken together, COR-D is concluded to inhibit the proliferation of B16F10 cells via intrinsic pathways and may be a potential anti melanoma agent.
RESULTS

**COR-D reduced cell viability in B16F10 cells**

COR-D inhibited proliferation of B16F10 cells in concentration dependent manner [Fig 6.1A]; this was further supported by 7-AAD positivity [Fig 6.1B]. IC50 value is 91.55±0.592 μM (24 h). Phase contrast microscopy showed morphological changes like cell shrinkage and nuclear condensation in treated cells. Taken together these results suggested that COR-D induced apoptosis in B16F10 cells.

![Fig 6.1: Cell growth inhibitory effect of COR-D in B16F10 cells.](image)

(A) B16F10 cells were treated with different concentrations (25-150 μM) of COR-D for 24 h and % of inhibition was determined by MTT assay. Each experiment was performed in triplicate. Percentage inhibition of the growth was calculated and expressed as mean ± SEM. *p < 0.01 implies significant difference in comparison to control group. (B) Cells were treated with IC50 concentration of COR-D for 24 h and pictorial view of apoptosis was recorded under phase contrast. (C) MTT data was supported by using 7-AAD positivity at different concentrations of COR-D.
**COR-D induced apoptosis in B16F10 cells involves mitochondrial pathway**

Cellular apoptosis normally involves intrinsic (mitochondrial) or extrinsic pathway or both. As COR-D treatment appeared to shift the cells towards apoptosis, involvement of mitochondria was evaluated through JC-1 staining. Accordingly, treated B16F10 cells were subjected to FACS analysis using JC-1, which showed mitochondrial membrane depolarization in a concentration-dependent manner [Fig 6.2].

![Image of FACS analysis showing mitochondrial membrane depolarization](image)

**Fig 6.2: Mitochondrial membrane depolarization following exposure of B16F10 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.

This suggested that the mitochondrial membrane potential was disrupted, resulting in cytosolic accumulation of monomeric JC-1. To validate the involvement of mitochondrial pathway, the expression levels of anti- and pro-apoptotic proteins Bcl-2, Bax, caspase 9, and caspase 3 were studied by Western blot analysis. Concentration-dependent decrease in Bcl-2 [Fig 6.3A] and increase in Bax [Fig 6.3B] were observed in the total cell lysate. Evaluation of the role of caspases in mitochondrial pathway thereafter showed that...
caspase 9 and caspase 3 were activated after COR-D treatment [Fig 6.3C and D]. These results confirmed that the cells undergo apoptosis through the mitochondria mediated pathway after treatment.

Fig 6.3: Expression profile of mitochondrial pro- and anti-apoptotic proteins in B16F10 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. Profiles and densitometric analysis of (A) Bcl2, (B) Bax, (C) caspase 9 and (D) caspase 3, in total cell lysate. Densitometric analyses 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 is a non-toxic and potent compound that inhibits tumors in BALB/c mice model**

We implanted a B16F10 xenograft into BALB/c mice to establish the antitumor activity of COR-D *in vivo*. Doses of 25 and 50 mg/kg/day of COR-D clearly inhibited tumor growth [*Fig 6.4A*] and reduced tumor volume after 10 days of consecutive treatment [*Fig 6.4C*].

![Fig 6.4: COR-D inhibits growth and induces apoptosis in the B16F10 xenograft in Balb/c mice model. (A) Normal, control and treated mice. (B) Effect of COR-D on the body weight of mice. (C) Isolated tumour of untreated and treated mice.](image)

Additionally, greater number of AnnexinV-FITC [*Fig 6.5C*] and PI-positive tumor cells [*Fig 6.5D*], were isolated from the treated mouse than the control, which signified the accumulation of more apoptotic cells. No visible sign of toxicity or mortality was noticed up to 200 mg/kg of COR-D administration for 20 days. The tumor weight increased for
Results

Effect of COR-D on B16F10 Cells

Untreated mice but deceased in the treated mice. COR-D mediated toxicity was not seen in the treated BALB/c mice group as judged by tumor weight measurements [Fig 6.4B] and tissue sections of tumor, liver, and kidney with respect to control [Fig 6.6]. Histological study revealed decreased microvascular density and mitotic index of the tumor cells.

Fig 6.5: COR-D induces apoptosis in \textit{in vivo} mice model. (A) and (B) show the tumor and tumor weight after initiation of treatment with COR-D, whereas the control receives only saline. (C) and (D) show the status of apoptotic cells in the tumor measured by annexin V-FITC and PI positivity in COR-D -treated and untreated mice as determined by flow cytometric analysis. The data are presented as mean ± SD from triplicate independent experiments.
Fig 6.6: Tissue sections of the livers, lungs, and spleen of untreated and COR-D treated Balb/c mice analyzed by H & E staining.
DISCUSSION

The present study reports the apoptotic effect of COR-D death in B16F10 melanoma cell line and in vivo mice model and the mechanisms involved in the induced activation of signaling cascades culminating in apoptotic cell. The results demonstrated that COR-D inhibits proliferation of B.6F10 cells in a concentration dependent manner. Further investigations showed that cultured B16F10 cells treated with COR-D exhibit morphological features of apoptosis, such as membrane shrinkage, nuclear fragmentation and chromosomal condensation.

The compound caused increase in PI positivity in a concentration dependent manner, which supports the contention that cell death after treatment occurs via apoptotic signaling pathway. In the complex signaling events of apoptosis, the ratio of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins in mitochondria changes, this results in loss of membrane potential and activation of caspases, leading to apoptosis (Yang et al., 1997). In the present study, concentration dependent decrease in Bcl-2 and increase in Bax were observed in COR-D treated B16F10 melanoma cells, which signifies that COR-D altered the Bax/Bcl-2 ratio and triggered the mitochondrial pathway of apoptosis. The activation of the caspase cascade indicates that the promotion of apoptosis in response to death inducing signals originated from cell surface receptors, mitochondria or endoplasmic reticulum. The present study revealed that COR-D induced elevation of some caspases, including caspase 3 and 9. Activation of initiator caspases such as caspase 9 in response to the pro-apoptotic signals activates caspase 3, the major effector caspase (Patel et al., 1996) which plays the central role in the initiation of apoptosis (Nicholson et al., 1995; Salvesen et al., 1999).

It was also found that COR-D inhibited the B16F10 tumor growth and increased the survival rate of mice. The reduction in tumor growth is well correlated with the decreased microvascular density and mitotic index of the tumor cells. Most importantly, the animals did not show any visual symptoms like hair loss, weight loss, diarrhoea and movement at doses of 25 and 50 mg/kg body weight. In conclusion, this study reveals that COR-D-
induced mitochondrial dysfunction is responsible for the induction of apoptotic cell death \textit{in vitro} and \textit{in vivo}. 
REFERENCES


References