STUDIES ON THE ANTITUMOR POTENTIAL OF BETULINIC ACID AND ASSOCIATED BIOCHEMICAL CHANGES IN MICE BEARING ASCITES DALTON’S LYMPHOMA

Ph.D. THESIS ABSTRACT
SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY

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Cancer is one of the leading causes of death worldwide. Cancer treatment aims at curing the disease or prolonging the patient’s life considerably. Cancer can be commonly treated by surgery, chemotherapy, radiation therapy, hormonal therapy and targeted therapy (including immunotherapy such as monoclonal antibody therapy) etc. In chemotherapy, hundreds of drugs of diverse chemical nature and implying different mechanisms of action have been used against a wide range of cancers. However, full use of these drugs has been limited due to development of various side effects in the hosts (Yao et al., 2007). Thus, in an attempt to overcome the side effects of chemotherapy the development of new drugs, using drugs in combination and the use of number of plants as well as animal-derived natural products have been tried. The use of natural resources has shown promise in this direction. Therefore, the development of novel, potent and non-toxic anti-cancer agents is a continuous effort of scientists all over the world.

Natural products, which are a rich source of compounds with enormous structural diversity, have been extensively explored in the field of drug discovery and have led to remarkable successes. This is particularly evident in the field of cancer therapeutics, where over 50% of the approved drugs discovered in the last two decades of the 20th century were of natural origin (Cragg and Newmann, 2005).

As the largest class of natural products, terpenoids consist of approximately 25,000 chemical structures thus, far with potential applications in the fragrance and flavour industries to the pharmaceutical and chemical industries (Gershenzon and Dudareva, 2007). Among the various triterpenes present in plant kingdom,
pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several biological and medicinal properties.

Betulinic acid (3b-hydroxy-lup-20(29)-en-28-oic acid) (BA) is one such naturally occurring pentacyclic lupane type triterpene, distributed widely throughout the plant kingdom. It was originally isolated from the bark of the white birch, *Betula pubescens*, from which it got its name (Tan et al., 2003). Other known sources of betulinic acid include *Ziziphus* spp. (Rhamnaceae) (Pisha et al., 1995), *Syzygium* spp. (Myrtaceae) (Kashiwada et al., 1998), *Diospyros* spp. (Ebenaceae) (Recio et al., 1995) and *Paeonia* spp. (Paeoniaceae) (Ikuta et al., 1995). The pure compound of betulinic acid appears as a white crystalline solid, melts at 295 - 297°C. It exhibits limited solubility in organic alcohols such as methanol, ethanol, chloroform, and ether and has low solubility in water, petroleum ether, dimethyl formamide and benzene (Yogeswari and Sriram, 2005). However, it is highly soluble in pyridine, DMSO and acetic acid.

In 1995, betulinic acid was reported as a highly selective growth inhibitor of human melanoma, neuroectodermal and malignant tumor cells and was reported to induce apoptosis in these cells (Pisha et al., 1995). While initial reports suggested that betulinic acid is selectively cytotoxic against melanoma cell lines, anticancer activity was subsequently reported against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia as well as several carcinoma, i.e. head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma (Qian et al., 2011).

Most of the anticancer research on betulinic acid has been carried out using cancer cell lines *in vitro* and the effects of betulinic acid *in vivo* needs to be further
researched. Moreover, the anticancer activity of betulinic acid against murine ascites Dalton’s lymphoma (DL) has not been studied.

Therefore, the present research study for the Ph.D. degree was undertaken to evaluate the antitumor effects of betulinic acid against murine ascites Dalton’s lymphoma, and to explore the possible mechanism of its antitumor activity with reference to apoptosis and biochemical changes.

In the present study, antitumor efficacy of betulinic acid (BA) in the tumor-bearing hosts was evaluated against murine ascites Dalton’s lymphoma (DL), using cisplatin (CDDP), a positive reference drug, which is considered one of the most effective anticancer chemotherapeutic agents used in clinical practices (Verma and Prasad, 2013).

The well-founded criteria for assessing the value of any anticancer drug is to examine the increase in life span of the hosts after the treatment. The results of the host’s survival data showed that betulinic acid is quite effective against DL, showing a significant increase in life span of the hosts as compared to that of control. The mean survival time of betulinic acid treated mice was significantly increased to about 50 days with ILS of about 150% which was quite comparable with that of cisplatin treatment showing ILS of about 177%

Cell viability/cytotoxicity study was determined by using MTT assay and trypan blue exclusion test. Cell viability/cytotoxicity study revealed that number of dead cells were increased significantly in mice after treatment with BA in a time dependent manner as compared to control which may result the increased survivability of the hosts. Further, as compared to DL cells, the spleen cells showed much higher viability at the corresponding time of BA treatment. It signifies that
betulinic acid was more cytotoxic to DL cells as compared to normal cells (spleen cells) in the host.

Surface morphological and internal structural changes in DL cells collected from the mice under different treatment conditions were done by light microscopy, scanning electron microscopy and transmission electron microscopy. Liver, kidney, and DL cells collected from control and treated groups of mice were used for the different biochemical analysis. Blood samples were collected from control and treated mice and used for different haematological determinations.

Apoptosis and/or necrosis are among the key mechanisms by which most compounds exert their cytotoxic effects, especially anticancer agents. The observations based on light microscopy, fluorescence microscopy (AO/EB staining), scanning electron microscopy and transmission electron microscopy are good reliable indicators for confirmation of apoptotic features. Betulinic acid depicted typical apoptotic features in DL cells like membrane blebbing, membrane shrinking, chromatin condensation, cytoplasmic vacuoles, nuclear fragmentation, apoptotic bodies etc which could be imperative in its antitumor activity.

Cell cycle analysis by flow cytometry is commonly performed to test whether the compound induced apoptotic cell death is associated with arrest of any of cell cycle phases. The flow cytometric study confirmed the presence of BA-induced apoptosis in DL cells by an increase in cells in S phase, with a decrease of cells in the G2/M phase indicating that betulinic acid causes cell cycle arrest at S phase in DL cells.

It has been indicated that mitochondria may serve as direct targets for betulinic acid (Fulda et al., 1999). In the present study BA-mediated changes in mitochondrial membrane potential ($\Delta \Psi_m$) was studied using rhodamine-123 dye by confocal laser
scanning microscopy and flow cytometry. Mitochondrial membrane potential decreased in DL cells in a time dependent manner after BA treatment as visible from reduced rhodamine 123 fluorescence intensity. A left shift of the peak from the mean value of control indicate enhanced cytochrome c release, opening of mitochondrial permeability transition pore with subsequent loss in mitochondrial membrane potential. Thus, the mitochondrial membrane potential is indeed compromised as the cells undergo programmed cell death.

Protein is an indicator of biological entity or activity. And hence in any biological reaction or estimation or bioprocess, protein analysis and quantification is done to determine the state of biological reaction or process. The time dependent decrease in the protein content in DL cells may involve changes in the rate of protein synthesis or decreased/increased uptake of protein in these cells or due to inhibition of protein synthesis.

Alterations in metabolism have been implicated in cancer, with the main focus on the Warburg effect, a phenomenon in which cancer cells upregulate glycolysis and lactate production while decreasing glucose contribution to the citric acid (TCA) cycle in the mitochondria, even in the presence of sufficient oxygen (Warburg, 1956; Lunt and Vander Heiden, 2011). Succinate dehydrogenase (SDH; EC 1.3.5.1) (succinate-coenzyme Q reductase, respiratory Complex II) catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Assay of SDH activity showed that as compared to the corresponding control, BA treatment showed time dependent decrease in SDH activity in DL cells and liver. Thus, targeting mitochondria as a cancer therapeutic strategy has gained momentum in the recent years.
Betulinic acid treatment caused an inhibition in the glycolytic enzymes activities such as hexokinase (HK; EC 2.7.1.1), pyruvate kinase (PK; EC 2.7.1.40) and lactate dehydrogenase (LDH; EC 1.1.1.27) thus, producing decreased glycolysis in DL cells, leading to antitumor effects. However, the increase of lactate dehydrogenase activity in blood serum and ascites fluid indicate leakage of lactate dehydrogenase from DL cells due to membrane damage/rupture. The reduction in the glycolytic capacity of tumor cells would restrict their ability to proliferate, invade adjacent tissues, and migrate to distant organs. This suggests that the attenuation of glycolysis in tumor cells may represent a useful strategy for preventing or stopping the development of cancer (Lopez-lazaro, 2007).

Glutathione (GSH) and GSH-related enzymes such as glutathione S-transferase (GST; EC 2.5.1.18), glutathione reductase (GR; EC 1.6.4.2) and glutathione peroxidase (GPx; EC 1.11.1.9) have been reported to be involved in intracellular defence mechanisms in the detoxification of peroxides, xenobiotics, hydroperoxides and drugs (Chasseaud, 1979; Meyer et al., 1998). In the present study changes in GSH and GSH-related enzymes (GST, GR and GPx) in liver, kidney, and DL cells of tumor-bearing mice were evaluated under different treatment conditions. GSH concentration did not change much in liver and kidney but decreased significantly in DL cells after betulinic acid treatment. GSH depletion can be seen to enhance the antitumour cytotoxicity. At the same time the decrease in GST, GR and GPx in DL cells could be one of the possible steps to cause decrease in the GSH level in DL cells, thus resulting in antitumor activity.

Molecular docking is frequently used to predict the binding orientations of small molecule drug candidates to protein/enzyme targets in order to in turn predict the affinity and activity of the small molecule. Docking results indicate that betulinic
acid and CDDP bind strongly to the active sites of the respective above mentioned enzymes, and this could be a possible reason for inhibition of these enzymes activities as noticed in enzyme assays. The docking results of BA and the above mentioned enzymes interaction showed an almost similar interaction in the active site of the enzymes as that of cisplatin, a known inhibitor, which causes inhibition in enzyme activity.

In present study haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cells volume (PCV), differential leukocytes count (DLC) were investigated which has prognostic importance in predicting the survival of mice with murine ascites Dalton’s lymphoma. The effect of betulinic acid administration increased the amount of haemoglobin, PCV, RBC counts, neutrophils, lymphocytes, while WBC counts were decreased. The results of present study clearly demonstrate that BA brought back haematological parameters to more or less to normal levels.

Histopathology is the microscopical examination of tissues from the body to spot characteristics of disease. In the present studies, liver and kidney sections from BA treated group did not show change in hepatic and renal architecture and the features were similar to that of control. Hence, the present study confirms the potent hepatoprotective nature of BA.

BA treatment did not show much change in plasma creatinine level as compared to control. CDDP treatment results in a significant increase in serum creatinine level as compared to that of control, thus indicating nephrotoxicity.

Liver is one of the prime target organs of any disease. Cancer results in hepatocytes damage, liver injury, and inflammation that lead to increased cell permeability and leakage of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Hence, elevated levels of AST and ALT in the plasma are
hallmarks of hepatic damage (Jain et al., 2012; Raghu et al., 2012). In BA-treated group, ALT and AST activities did not change much as compared to control. In comparison, CDDP treatment resulted in a significant increase in AST and ALT activities, thus, indicating hepatotoxicity.

Cholesterol is a neutral lipid that plays an essential role in the maintenance of the integrity of biological membranes and serves as a precursor in the synthesis of many endocrine mediators. It serves as a precursor for the synthesis of steroid hormones, bile acids, and vitamin D (Russel and Setchell, 1992). As compared to tumor-bearing control mice, serum cholesterol levels were significantly decreased in BA-treated mice. Similarly, CDDP treatment also resulted in a significant decrease in cholesterol level was observed as compared to control.

Based on the findings from various aspects of studies undertaken, following important conclusions may be derived:

- Betulinic acid is quite effective against murine ascites Dalton’s lymphoma showing a significant increase in life span of the hosts. Further, betulinic acid is found to be more cytotoxic to DL cells than normal cells i.e. spleen cells.

- Various apoptotic features observed in tumor cells after betulinic acid treatment could be an important step in developing the antitumor activity in the host. Cell cycle analysis revealed that betulinic acid treatment caused an increase in the cells in the S phase which may suggest that it prevents DL cells from replicating further, which may induce tumor cell death and favouring towards hosts survivability.

- Betulinic acid treatment caused a time-dependent decrease in mitochondrial membrane potential which may also add to its antitumor effects against DL cells. The decrease in SDH activity in DL cells after betulinic acid treatment
may also play a role in the development of mitochondrial dysfunction contributing to tumor cells death.

- Betulinic acid treatment caused a decrease in the GSH levels in DL cells which may lead to a decrease in the protective ability of the cells thereby may become more prone to oxidative stress and cell injury.

- Betulinic acid treatment caused an inhibition in the glycolytic enzymes activities such as hexokinase and pyruvate kinase thus, resulting decreased glycolysis in DL cells, leading to antitumor effects. Molecular docking findings indicate that betulinic acid binds strongly to the above mentioned enzymes, which could be one of the possible reasons for inhibition of these enzymes activities.

- The decrease in LDH activity in DL cells and a simultaneous increase in the blood serum and ascites fluid after betulinic acid treatment may indicate release of LDH from DL cells due to membrane damage.

- The observed betterment or no changes in the different haematological parameters after betulinic acid treatment may suggest that it shows no hematotoxicity in the hosts. Betulinic acid treatment also showed no/minimal toxicity in liver and kidney, which was further confirmed by assaying the different liver function and renal function tests. Serum cholesterol levels were also found to decrease after betulinic acid treatment.

Given the fact that no serious adverse side effects were observed following BA treatment, BA emerges to be an attractive cytotoxic agent for the treatment of various types of cancer and murine ascites Dalton’s lymphoma in particular.
REFERENCES


