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BETULINIC ACID-MEDIATED INDUCTION IN APOPTOSIS AND INHIBITION IN LACTATE DEHYDROGENASE ACTIVITY IN MURINE ASCITES DALTON'S LYMPHOMA

Anamika Bhaumik[1]*, Arun Bahadur Gurung[2], Atanu Bhattacharjee[2] and Surya Bali Prasad[1]

[1]Cell and Tumor Biology Laboratory, Department of Zoology, North-Eastern Hill University, Shillong-793022, Meghalaya, India.

[2]Computational Biology Laboratory, Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong-793022, Meghalaya, India.

***Corresponding Author: Anamika Bhaumik**

Cell and Tumor Biology Laboratory, Department of Zoology, North-Eastern Hill University, Shillong-793022, Meghalaya, India.

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ABSTRACT

Betulinic acid is a pentacyclic triterpenoid originally isolated from the bark of the white birch *Betula pubescens*, from which it got its name. Antitumor potential of betulinic acid against murine ascites Dalton's lymphoma (DL) has been established. The light, fluorescence and transmission electron microscopy based study showed that DL cells treated with betulinic acid depicted typical apoptotic features which could be imperative in its antitumor activity. Betulinic acid treatment also caused a significant decrease in lactate dehydrogenase (LDH) activity in DL cells. Further, molecular docking study revealed the strong binding affinity of betulinic acid with LDH which could be noteworthy in resulting decreased LDH activity. Based on these findings, it may be suggested that along with the induction in apoptosis, lactate dehydrogenase could be a possible target for betulinic acid-mediated antitumor activity in DL-bearing mice, since its inhibition by betulinic acid may decrease energetic and anabolic supply to cancer cells, which could be one of the important factors to reduce the metastatic and invasive potential of DL cells.

KEYWORDS: Betulinic acid, Apoptosis, Dalton's lymphoma, Lactate dehydrogenase, Molecular docking.

INTRODUCTION

Natural products are being used extensively for treatment of a myriad of diseases including various types of cancers.[1] For cancer chemotherapy, the phytochemicals and their synthetic analogues are among the most widely used drugs.[2,3] Phytochemicals and microbial extracts from various sources are routinely screened for biological activities, and it is estimated that 20% to 25% of new drugs are derived from natural products or their synthetic analogues.[1, 4, 5]

Plant triterpenoids from lupane series which exhibit potent antitumor activity have been the focus of research interest.[6] Betulinic acid (3b-hydroxy-lup-20(29)-en-28-oic acid) (BA) (Fig. 1) is one such pentacyclic triterpenoid, lupane derivative which was originally isolated from the bark of the white birch *Betula pubescens*, from which it got its name. BA is also found in other plants sources such as *Tryphyllum peltaum*, *Ancistrocladus heyneaus*, *Zizyphus joazeiro*, *Diospyoros leucomelas*, *Tetracera boliviana* and *Syzygium formosanum*.[7] Initially, it was found to be a melanoma specific cytotoxic agent that inhibited the growth of human melanoma tumors in athymic mice.[8] Later, it was found that the antitumour activity of BA was not only restricted to melanoma but it was found to be active on non-melanoma cancers also and it showed a significant *in vivo* antitumour activity on ovarian carcinoma IGROV-1 xenografts.[9] BA has been shown to induce apoptosis in several cancer cell lines such as neuroblastomas and glioblastomas through the mitochondrial activation pathway.[10, 11] The cytotoxicity research on BA showed that it had selective cytotoxicity on tumor cell lines but not on normal cells,[9] suggesting that it may have potential for its development as a potent cancer therapeutic agent.[12]

Lactate dehydrogenase (LDH; EC 1.1.1.27) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+.[13] It is one of the important enzymes of glycolysis and it is released into the surrounding culture medium upon cell damage or lysis of the tissue. Therefore, assaying the level of LDH may indicate a measurement of cytotoxicity.

Docking is one of the important computational tools for identifying the structural modelling and predicting the activity of any potential drug. It is the most commonly

employed technique for calculating binding affinities and predicting binding sites.[14] Earlier, it has been reported by us that BA has potent antitumor activity against murine ascites Dalton's lymphoma.[15]

Based on this background of research findings, present study was intended to further analyze the mechanism of possible antitumor activity with reference to apoptosis and LDH activity in DL cells. Cisplatin (*cis*–diamminedichloroplatinum (II) (CDDP), a potent cancer chemotherapeutic agent, was used as a positive control in the study.[16, 17]

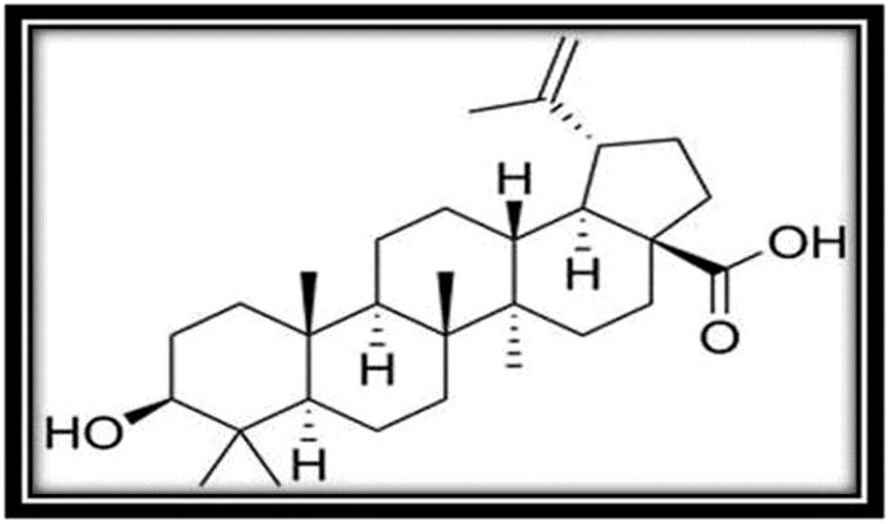

Figure 1: Chemical structure of betulinic acid.

MATERIALS AND METHODS

Chemicals

Betulinic acid (≥98% purity), acridine orange and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, Mo, USA. NADH (Nicotinamide adenine dinucleotide), sodium pyruvate and all other chemicals used in the experiments were of analytical grade and purchased within the country. Cisplatin solution (1 mg/ml of 0.9% NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India.

Murine tumor model and its maintenance

Ascites Dalton's lymphoma (DL) has been commonly used murine malignant tumor in the screening of anticancer potentials of chemical agents as well as animals and plants extracts.[18] It initially originated in the thymus gland of DBA/2 mouse at the National Cancer Institute, Bethesda, MD, USA in 1947 and subsequently an ascites form was developed by repeated intraperitoneal (i.p.) transplantation of the tumor.[19] Later, in India probably this tumor cell line was first procured in 1951 by Cancer Research Institute, Mumbai.

Inbred Swiss albino mice colony was maintained under conventional laboratory conditions at room temperature (20±2ºC) with free access to food pellets (Amrut Laboratory, New Delhi) and water *ad libitum*, keeping 5-6 animals in a propylene cage. Ascites Dalton's lymphoma (DL) tumor was maintained *in vivo* in 10-12 weeks old mice of both sexes by serial intraperitoneal (i.p.) transplantation of viable tumor cells to the animals as per the established procedure.[20] Tumor-transplanted hosts usually survived for 19-21 days. Following tumor transplantation, an increase in abdomen size and body weight with sluggish movement of the animals was noted from 3rd-4th day onwards which was an early sign of tumor development.

The maintenance, use of the animals and the experimental protocol of the present study was approved by the Institutional Animals Ethical Committee, North-Eastern Hill University, Shillong.

Drug treatment schedule

Betulinic acid was dissolved in dimethyl sulfoxide (DMSO) at a dose of 10 mg/ml before use. It was diluted in phosphate-buffered saline to get the desired concentration and based on the earlier reports,[21] the therapeutic dose of betulinic acid was selected as 10 mg/kg body weight and 0.25 ml of the diluted drug was injected (i.p.) into mice. The day of tumor transplantation was taken as day '0'. Tumor-transplanted mice were randomly divided into three groups consisting of 10 mice in each group. Group-I mice served as tumor-bearing control and received phosphate-buffered saline only. Group-II mice were injected with betulinic acid (i.p., 10 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation. Group-III mice were administered with cisplatin (i.p., 2 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation. Two animals were sacrificed by cervical dislocation after 24, 48, 72 and 96 hours interval following last treatment with the drug (i.e. on the 11th, 12th, 13th and 14th day post tumor transplantation) and the tumor cells were collected for different studies. The experiments were repeated three times.

Light microscopy (LM) study

Animals in different treatment groups were sacrificed by cervical dislocation. The ascites tumor was collected and centrifuged at 1000xg for 5 minutes at 4°C, washed in PBS (0.15M NaCl, 0.01M sodium phosphate buffer, pH 7.4). The cell pellet was resuspended in PBS (1: 4) and a drop of the cell suspension was taken on a clean slide and spread gently. The cells were fixed in absolute methanol for 15 minutes and stained the following day with Leishman's stain. The cells in different groups were thoroughly examined and photographed for any morphological changes.

Flourescence based apoptosis study

Flourescence based apoptosis was determined in DL cells collected from mice under different treatment conditions using acridine orange-ethidium bromide (AO/EB) staining method following the method of Shylesh et al.[22] as previously used in the lab.[23] After treatment, DL cells were collected from mice at different time intervals (24, 48, 72 and 96 hours). The cells were washed twice with PBS and treated with AO/EB (100µg/ml PBS of each dye) for 5 minutes and gently washed with PBS. The cells in different treatment groups were thoroughly examined under a fluorescence microscope and photographed (A1000IS - canon).

Transmission electron microscopy (TEM) study

DL cells pellet collected from mice under different experimental conditions were used for transmission electron microscopy study. The ascites tumor was collected from the peritoneal cavity using a glass syringe with a disposable needle, centrifuged (1000xg, 4ºC, 8-10 minutes) and the cells pellets were washed with PBS. DL cells pellets were fixed in 3% glutaraldehyde (prepared in 0.1 M cacodylate buffer) for 2 hours at 4ºC. After fixation, the cells were washed with 0.1 M cacodylate buffer. Then the cells pellet were broken into small pieces and post-fixed in 1% osmium tetroxide for 15 minutes at 4ºC. The post-fixed samples were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95%, twice at each concentration for 15 minutes each) at room temperature and finally kept in dry acetone (prepared by adding $CuSO_4$ crystals in excess to absolute acetone and filtered). These were then kept in propylene oxide, twice for 1 hour at room temperature and at different ratio of propylene oxide and embedding medium (3:1, overnight), (1:1, 1 hour), (1:3, 1 hour) and in pure embedding medium twice for 2 hours at 50ºC. The embedding medium (araldite) was prepared by thoroughly mixing of araldite Cy212, dodecenyl succinic anhydride (DDSA), tridimethylamino methyl phenol (DMP-30) and 1.0 ml of dibutylphdyhalate. Ultrathin sections (60-80 nm) were cut in an ultra microtome (ultratome-RMC, MTX, USA) and collected on copper grids. These were stained with lead citrate and uranyl acetate (1:1 v/v). Then viewing was done in the transmission electron microscope (Jeol, TEM) operated at a voltage of 80 KV. The grids/sections were scanned and photomicrographs were taken after observing thoroughly different portions of the sections collected on at least four grids.

Lactate dehydrogenase (LDH; EC 1.1.1.27) assay

Lactate dehydrogenase (LDH) activity was assayed in DL cells following the method of Bisswanger (2000).[24] Total assay volume of 1.0 ml contained 9.4 ml 0.1 M potassium phosphate (pH 7.0), 0.2 ml 0.01 M NADH, and 0.2 ml 0.1 M pyruvate. The overall reaction (maintained at 25ºC) was started by adding 0.02 ml of the enzyme sample and the decrease in absorbance at 340 nm was monitored. The enzyme activity was calculated using the extinction coefficient (E_{340} = $6.3x10^3$ L mol^{-1} cm^{-1}). Specific activity was expressed in units per mg of protein.

Molecular docking studies

The structures of betulinic acid (CID64971) and cisplatin (CID2767) were retrieved from PubChem Database (https://pubchem.ncbi.nlm.nih.gov/). The structures were then optimized using MMFF force field with optimization parameters such as 500 steps of steepest descent algorithm and convergence criterion of 10e-7.[25] The optimized structures were used for molecular docking studies.

The three dimensional structure of lactate dehydrogenase, LDH-A (4JNK)[26] was obtained from Protein Data Bank (PDB) (www.rcsb.org). The steps for preparation of proteins included (a) removal of heteroatoms (water, ions), (b) addition of polar hydrogens, and (c) assignment of Kollman charges. The active sites were defined by considering grid boxes of appropriate sizes around the bound co-crystal ligands encompassing the active site residues (Table 1). The compounds were docked against LDH using AutoDock4.2.[27] The parameters for unknown atom type Pt (II) was included in the AutoDock parameter file.[28, 29] The docking experiment was performed using Lamarckian Genetic Algorithm, with an initial population of 250 randomly placed individuals, a maximum number of 10^6 energy evaluations, a mutation rate of 0.02 and a crossover rate of 0.8. One hundred independent docking runs were performed for each compound. Conformation were clustered considering root mean square deviation (RMSD) cut-off of 2.0 Å and the lowest free energy of binding (ΔG) represent the most favourable conformation. The most favourable binding conformation was selected and evaluated for molecular interaction with their receptors using Molegro Molecular viewer vs 2.5.[30]

Table 1. Particulars of grid box dimensions considered for molecular docking.

Protein	Co-crystal ligand	Grid box dimensions		
		No. of grid points	Center (xyz coordinates)	Grid point spacing
Lactate dehydrogenase	Lactic Acid (LAC)	50× 50× 50	45.941, 21.701, 47.229	0.375

Statistical analysis

The data were expressed as mean ± SD. Comparisons between the control and treated groups were made by one way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with Tukey multiple comparison tests, $p \leq 0.05$ was considered as statistically significant in all the cases.

RESULTS

Light microscopy (LM) study

Control DL cells were roundish in shape. In BA treated mice, DL cells showed the appearance of membrane blebbing and folding during 24 hours to 72 hours of treatment, while at 96 hours of treatment, cells with severe membrane rupture along with cytoplasmic vacuoles could be noticed. In CDDP treated mice, DL cells showed the appearance of membrane blebbing and folding with severe membrane rupture was observed at 96 hours of treatment (Fig. 2).

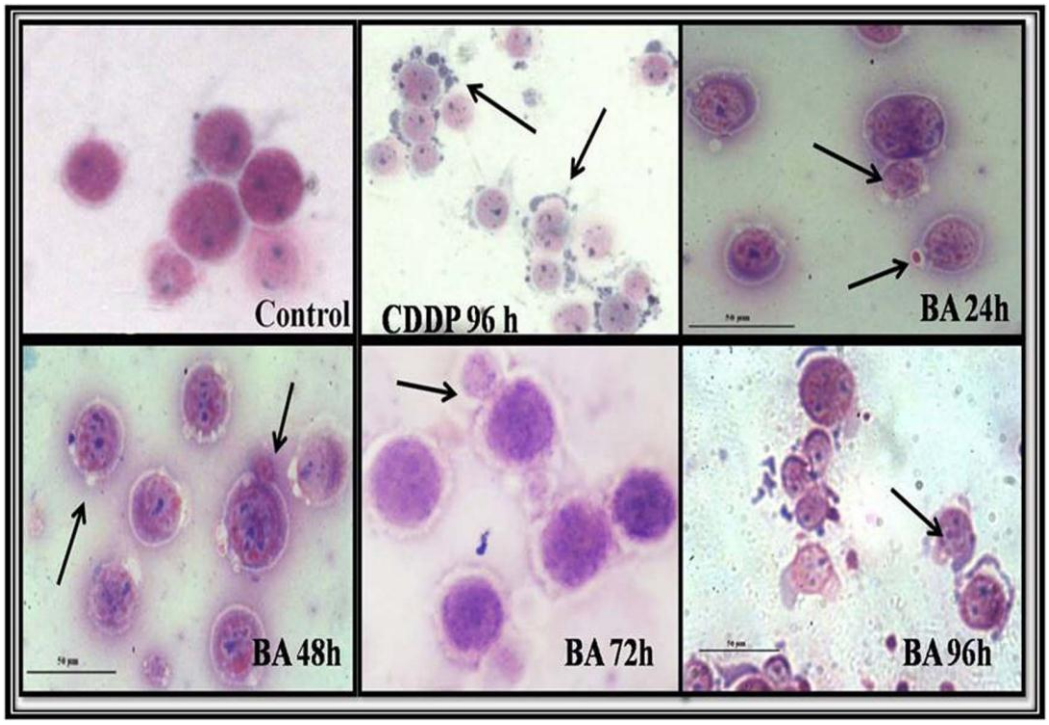

Figure 2: Light micrographs of DL cells from different treatment groups. Arrows show apoptotic features with membrane blebbing/folding, fragmented nucleus and vacuoles inside the cytoplasm. BA= Betulinic acid; CDDP= Cisplatin.

Flourescence based apoptosis study

Acridine orange (AO) is a vital dye that stains both live and dead cells, whereas, ethidium bromide (EB) stains only those cells that have lost membrane integrity. Cells stained green represent viable cells, whereas orange/red stained cells represent apoptotic cells. Control DL cells were round in shape with uniform green fluorescence indicating viable cells while cisplatin treatment after 96 hours showed many apoptotic nuclei with membrane blebbing and fragmented nuclei. In betulinic acid treated mice, DL cells illustrate the appearance of membrane blebbing and folding at 24 hours. Chromatin condensation and cell membrane abnormality with fragmented nuclei were observed at 48 hours of BA treatment. After 72 hours of BA treatment, cells with severe membrane rupture with many fragmented nuclei and cytoplasmic vacuoles were noted. BA treatment of mice for 96 hours showed damages in plasma membrane with scattered fragmented nuclei and apoptotic bodies outside the cells (Fig. 3). The results suggest that BA was able to induce marked apoptosis in DL cells.

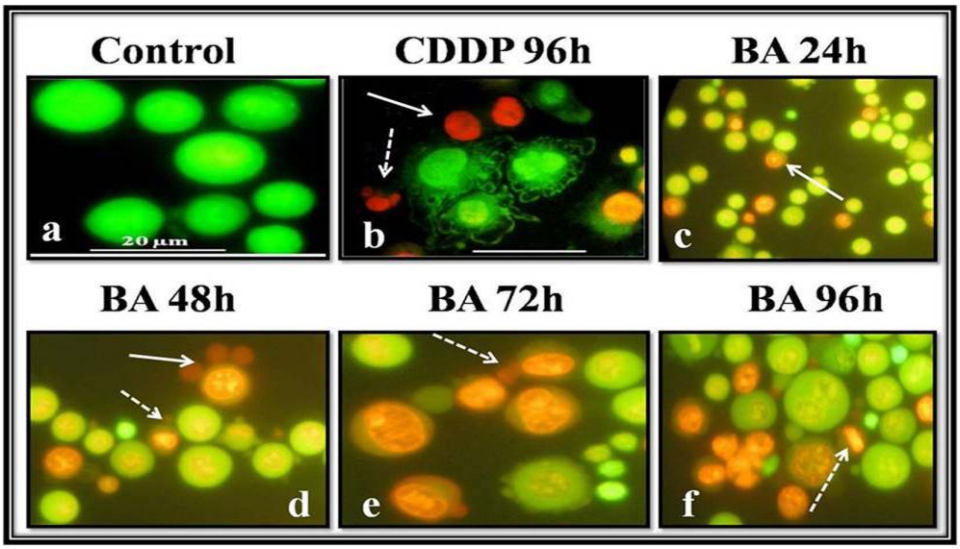

Figure 3: Flourescence based determination of apoptosis in DL cells using AO/EB staining method; (a) control; (b) cisplatin; (c-f) betulinic acid treatment for 24-96 hours. Cells stained green represent viable cells, whereas orange/red stained cells represent apoptotic cells. Regular arrow represents apoptotic features with membrane blebbing/folding; dotted arrow represents fragmented nucleus. Abbreviation of BA and CDDP in the figure denotes betulinic acid and cisplatin respectively.

Transmission electron microscopy (TEM) study

The control cells showed large nuclei, uniform chromatin, abundant euchromatin, normal organelles structure and a smooth plasma membrane. Cisplatin (CDDP) treatment at 72 hours showed the appearance of chromatin condensation, vacuolization of cytoplasm and disintegration/distortion of plasma membrane. BA treatment of mice at 24 hours showed the appearance of chromatin condensation and cytoplasmic vacuoles in DL cells. Membrane disorganization, fragmented nuclei and cytoplasmic vacoules were observed during 72 hours of BA treatment which ultimately may lead to lysis of tumor cells (Fig. 4).

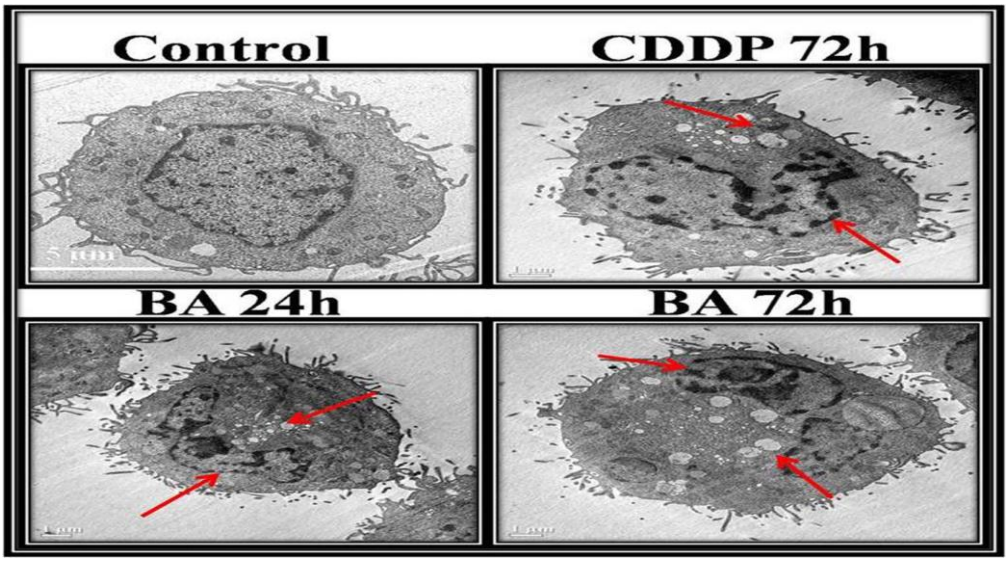

Figure 4: Ultrastructural features of DL cells. Tumor-bearing control showing more or less rounded shape, normal nucleus with microvilli like processes over the cell surface. Compared with normal microstructure, betulinic acid (BA) treatment of mice at 24 hours and 72 hours showed nuclear shrinkage, cytoplasmic vacuolization, disintegration in the cells surface and cell membrane distortion. Cisplatin (CDDP) treatment also showed almost similar ultrastructural changes like betulinic acid. BA- Betulinic acid; CDDP- cisplatin.

Lactate dehydrogenase (LDH; EC 1.1.1.27) activity

As compared to corresponding control, betulinic acid treatment resulted in a significant decrease in LDH activity in DL cells in a time dependent manner (24-96 hours). In case of cisplatin treatment also, a significant decrease in LDH activity was observed in DL cells in a time dependent manner (24-96 hours) as compared to control (Fig. 5).

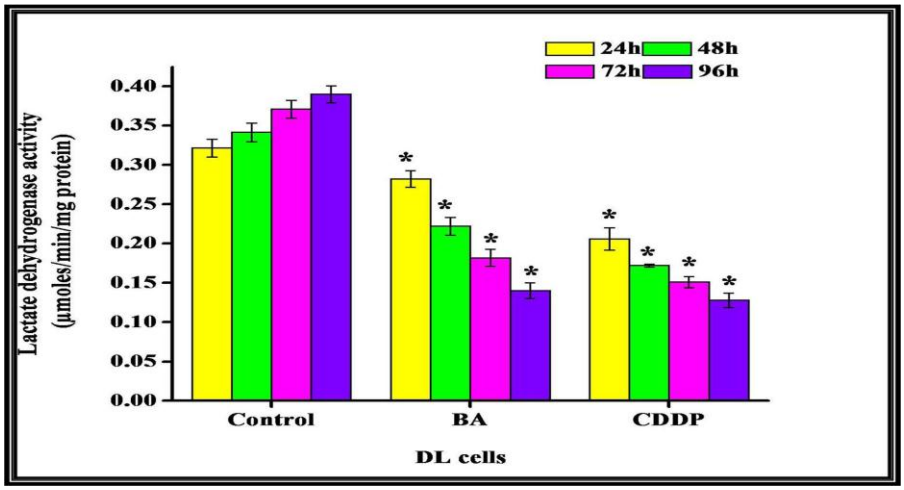

Figure 5: LDH activity in DL cells of tumor-bearing mice after treatment with betulinic acid and cisplatin respectively. The results are expressed as mean ± SD., ANOVA, n=3, *p≤0.05 as compared to corresponding control group. BA-betulinic acid, CDDP- cisplatin.

Molecular docking

The inhibitory activity of BA on LDH was analyzed through binding of betulinic acid and cisplatin to LDH by docking simulations. During docking, cisplatin, a known inhibitor of LDH, was used as a reference ligand, for comparison of binding affinity with betulinic acid. BA was capable of docking into the active site of LDH enzyme. This compound fitted well in the active site of LDH, similar to that of cisplatin as shown by binding affinity. The docking results showed that betulinic acid and cisplatin bind to lactate dehydrogenase with ΔG of -8.42 kcal/mol and -3.48 kcal/mol respectively. The binding modes analysis of betulinic acid with lactate dehydrogenase reveals that it formed three hydrogen bonds with Asp140 and Ile141 of distances 2.97, 3.52 and 3.31 Å respectively. The other non-bonded contacts involve residues-Arg105, Val109, Asn137, Pro138, Glu191, His192, Gly193, Asp194, Tyr238, Ile241 and Leu322 (Fig. 6A). Cisplatin formed three hydrogen bonds with Asp140, Glu191 and His192 of distances 2.69, 3.08 and 2.81 Å respectively. The non-bonded contacts involve residues-Asn137, Pro138, Val139, Ile141 and Leu322 (Fig. 6B).

Table 2. The binding free energy of the compounds obtained from molecular docking experiment.

Protein	Compounds	Binding Free Energy (ΔG) [kcal/mol]
Lactate Dehydrogenase	Betulinic acid	-8.42
	Cisplatin	-3.48

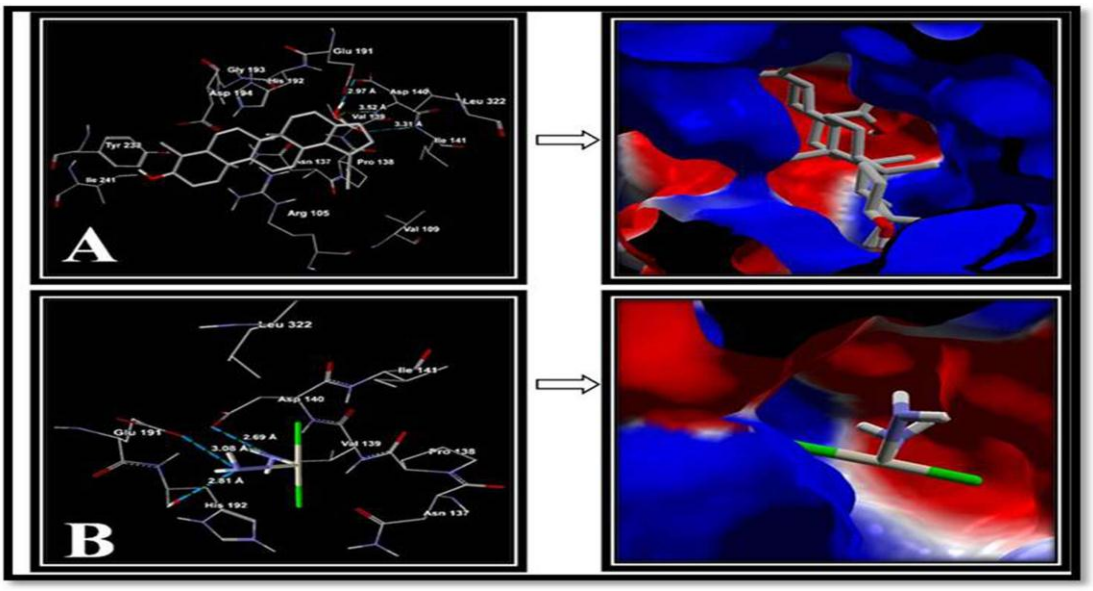

Figure 6: The binding modes and molecular interaction of betulinic acid and cisplatin with lactate dehydrogenase (A) Betulinic acid and lactate dehydrogenase (B) Cisplatin and lactate dehydrogenase.

DISCUSSION

Natural products have been a source of medicinal agents for thousands of years.[31] Several side effects have been associated with the cancer chemotherapy treatment. Therefore, the development of alternative potent therapeutic agents having minimal or no side effects could be of greater interest.[32] BA (Fig. 1) is a novel antineoplastic agent showing more cytotoxicity against melanoma and neuroectodermal tumor cells. The findings from the present studies suggest that BA triggers apoptosis in murine ascites Dalton's lymphoma and the findings are in agreement with other reports.[33] BA inhibits the growth of cancer cells effectively in lung, colon, prostate and ovary carcinomas without any effect on normal cells.[8, 9, 33] Apoptotic characteristics were observed in treated DL cells by the appearance of cell shrinkage, cell shortening, membrane blebbing, nuclear condensation and/or the presence of membrane-bound apoptotic bodies. Apoptosis has always been regarded as an ideal way to destroy damaged cells, and an agent that can induce apoptosis is preferable in the management and therapy of cancer.[34, 35]

Light microscopy revealed that the treatment of DL cells with BA for 24-96 hours induced marked changes in cell morphology. The cells treated with BA showed nuclear fragmentation, membrane rupture, membrane blebbing, appearance of vacuoles inside the cytoplasm and formation of apoptotic bodies (Fig. 2). The remarkable phenotypic alterations in apoptotic cells may be caused by the destruction of the normal nuclear architecture.[36, 37]

Apoptosis was further confirmed in DL cells using acridine orange-ethidium bromide (AO/EB) staining. Live cells have a normal green nucleus, whereas the apoptotic cells display condensed red nucleus.[38] Control DL cells were round in shape with uniform green fluorescence while cisplatin treatment for 96 hours showed many apoptotic nuclei with membrane blebbing and fragmented nuclei. In betulinic acid treated mice, DL cells illustrated the appearance of membrane blebbing, fragmented nuclei and apoptotic bodies. The increased percentage of red/ orange coloured cells (after AO/EB staining) observed in the BA treated conditions due to membrane breakage indicated that BA could induce apoptosis (Fig. 3).

Transmission electron microscopy (TEM) has been commonly used to study the ultrastructure of cells.[39] The application of TEM is also used in the detection of associated cellular micro-lesions in apoptosis and cell cycle arrest.[40, 41] In addition, the ultrastructural features in DL cells showed that the control cells had large nuclei, clear nucleoli, uniform chromatin, abundant euchromatin, normal organelle structure and a smooth plasma membrane structure. Cisplatin treatment at 72 hours showed the appearance of chromatin condensation, vacuolization of cytoplasm and disintegration/distortion of the plasma membrane. BA treatment of mice for 24 hours showed the appearance of chromatin condensation and cytoplasmic vacuoles in DL cells. Membrane disorganization, fragmented nuclei and vacuolization in the cytoplasm were observed during 72 hours of treatment which finally leads to lysis of the tumor cells (Fig. 4).

Lactate dehydrogenase plays an important role in glycolysis and glycolytic activity in tumor cells, popularly known as Warburg effect, suggests that cancer cells dependence on glycolytic energy progressively increases as malignant transformation occurs.[42] 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.[43] LDH is located in the cytosol, and is released into the surrounding culture medium upon cell damage or lysis. Recent observations showed that attenuation of LDH reduced the glycolytic metabolism of cancer cells and produced antitumor effects in animals.[44,45] The inhibition of LDH activity may represent a relatively nontoxic approach to interfere with tumor growth.[45] BA treatment caused a significant decrease in LDH activity in DL cells in a time dependent manner (24-96 hours) (Fig. 5). Cisplatin (CDDP) treatment also resulted in a significant time dependent decrease in LDH activity in DL cells (24-96 hours). This inhibitory effect on LDH activity may indicate inhibition/decreased glycolysis in DL cells. Multiple studies on various cancer cell lines have shown that attenuation of LDH in tumor cells increases apoptosis[45,46] and reduces migration and invasion ability[47,48] demonstrating its use as a potential therapeutic target. Therefore, it is suggested that LDH inhibition could be a well-tolerated therapy that will impede tumor growth and metastasis.

Docking is frequently used to predict the binding orientations of small molecules drug candidates to protein targets in order to in turn predict the affinity and activity of the small molecule. The receiving molecule that primarily binds to a small molecule or another protein or a nucleic acid is called receptor. A molecule that forms the complementary partner in the docking process is called ligand. AutoDock4.2 was used for the molecular docking studies of LDH with BA and CDDP.[27] Docking results indicate that BA and CDDP strongly bind to the active site of LDH with binding energies of -8.42 and -3.48 (ΔG) [kcal/mol] respectively (Table 2). The docking results of BA-LDH interaction showed an almost similar interaction in the active site of LDH as that of cisplatin, a known inhibitor of LDH, which caused inhibition in LDH activity (Fig. 6A and 6B). Thus LDH enzyme may be considered as a potential target for new anticancer agents, since its inhibition may cut cancer energetic and anabolic supply, thus reducing the metastatic and invasive potential of cancer cells.[47, 48] It is suggested that the anti-proliferative activity of BA

may involve apoptosis and inhibition of LDH activity in DL cells.

CONCLUSION

The findings from the results of present studies ascertained that the antitumor potential of BA against murine ascites Dalton's lymphoma may involve induction in various apoptotic features and decreased LDH activity in DL cells. Further, the results from molecular docking studies showed that there is strong interaction between LDH and BA, which might cause an inhibition in LDH activity. Thus, this enzyme may be considered as a potential target for betulinic acid, since its inhibition may decrease energetic and anabolic supply to tumor cells. Given the fact that no adverse side effects following BA treatment have been reported, BA appears to be an attractive cytotoxic agent for the treatment of various types of cancers and murine ascites Dalton's lymphoma in particular.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. J Nat Prod, 2003; 66: 1022–37.
2. Kowalski RJ, Giannakakou P, Gunasekera SP, Longley RE, Day BW, Hamel E. The microtubule-stabilizing agent discodermolide competitively inhibits the binding of paclitaxel (taxol) to tubulin polymers, enhances tubulin nucleation reactions more potently than paclitaxel, and inhibits the growth of paclitaxel-resistant cells. Mol Pharmacol, 1997; 52(4): 613–22.
3. Jordan MA. Mechanism of action of antitumor drugs that interact with microtubules and tubulin. Curr Med Chem Anticancer Agents, 2002; 2(1): 1–17.
4. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J Nat Prod, 1997; 60: 52–60.
5. Proudfoot JR. Drugs, leads, and drug-likeness: an analysis of some recently launched drugs. Bioorg Med Chem Lett, 2002; 12(12): 1647–50.
6. Pokrovskii AG, Shintyapina AB, Pronkina NV, Kozhevnikov VS, Plyasunova OA, Shul'ts EE, Tolstikov GA. Activation of Apoptosis by Derivatives of betulinic acid in human tumor cells *in vitro*. Dokl Biochem Biophys, 2006; 407(1): 94–7.
7. Vlietinck AJ, De Bruyne T, Apers S, Pieters LA. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Med, 1998; 64(2): 97–109.
8. Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, Beecher CWW, Fong HHS, Kinghorn AD, Brown DM, Wani MC, Wall ME, Hieken TJ, DasGupta TK, Pezzuto JM. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med, 1995; 1: 1046–51.
9. Zuco V, Rhigetti SC, Cleris E, Marchesi L, Passserini CG, Formelli F. Selective cytotoxicity of betulinic acid on tumor cell lines but not on normal cells. Cancer Lett, 2002; 175(1): 17–25.
10. Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Marcus E. Peter, Debatin KM. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res, 1997; 57: 4956–64.
11. Jeremias I, Steiner HH, Benner A, Debatin KM, Herold-Mende C. Cell death induction by betulinic acid, ceramide and TRAIL in primary glioblastoma multiforme cells. Acta Neurochir, 2004; 146(7): 721–9.
12. Hata K, Hori K, Ogasawara H, Takahashi S. Anti-leukaemia activities of Lup-28-al-20(29)-en-3-one, a lupane triterpene. Toxicol Lett, 2003; 143(1): 1–7.
13. Burgner JW, Ray WJ Jr. Acceleration of the NAD cyanide adduct reaction by lactate dehydrogenase: the equilibrium binding effect as a measure of the activation of bound NAD. Biochemistry-Us, 1984; 23(16): 3620–26.
14. Bajorath J. Integration of virtual and high-throughput screening. Nat. Rev. Drug Discovery, 2002; 1: 882-94.
15. Bhaumik A, Prasad SB. Studies on the antitumor potentials of betulinic acid against murine ascites Dalton's lymphoma. Int J Basic Clin Pharmacol, 2016; 5(4): 1664-71.
16. Ajith TA, Janardhanan KK. Cytotoxic and antitumor activities of a polypore macrofungus, *Phellinus rimosus* (Berk) Pilat. J. Ethnopharmacol, 2003; 84(2): 157-62.
17. Verma AK, Prasad SB. Antitumor effect of blister beetles: An ethno-medicinal practice in Karbi community and its experimental evaluation against a murine malignant tumor model. J Ethnopharmacol, 2013; 148(3): 869-79.
18. Prasad SB, Giri A, Khynriam D. Studies on the chromosomes of murine Dalton's lymphoma cells and the effect of cisplatin on chromosomes of these cells and bone marrow cells *in vivo*. Cytologia, 1998; 63(4): 405-13.
19. Goldie H, Felix MD. Growth characteristics of free tumor cells transformed serially in the peritoneal fluid of the mouse. Cancer Res, 1951; 11(2): 73-80.

20. Verma AK, Prasad SB. Changes in glutathione, oxidative stress and mitochondrial membrane potential in apoptosis involving the anticancer activity of cantharidin isolated from redheaded blister beetles, *Epicauta hirticornis*. Anticancer Agents Med Chem, 2013; 13(7): 1096-114.
21. Alitheen NB, Mashitoh AR, Yeap SK, Shuhaimi M, Abdul Manaf A, Nordin L. Cytotoxic effect of damnacanthal, nordamnacanthal, zerumbone and betulinic acid isolated from Malaysian plant sources. Int Food Res J, 2010; 17: 711-9.
22. Shylesh BS, Nair SA, Subramoniam A. Induction of cell specific apoptosis and protection from Dalton's lymphoma challenge in mice by an active fraction from *Emilia Sonchifolia*. Indian J Pharmacol, 2005; 37(4): 232-7.
23. Verma AK, Prasad SB. Bioactive component, cantharidin from *Mylabris cichorii* and its antitumor activity against Ehrlich ascites carcinoma. Cell Biol Toxicol, 2012; 28(3): 133-47.
24. Bisswanger H. Practical enzymology, Wiley-VCH Verlag & GmbH & Co. KGaA, Weinheim: 2000; 1-255.
25. Halgren TA. Merck molecular force field. I. Basis, form, scope, parameterization and performance of MMFF94. J Comput Chem, 1996; 17(5): 490-519.
26. Rong S, Xin L, Yuanyuan L, Xun Z, Xinru L, Xiaoyu L, Zheng S, Jinku B. Screening of novel inhibitors targeting lactate dehydrogenase A via four molecular docking strategies and dynamics simulations. J Mol Model, 2015; 21: 133.
27. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 2009; 30: 2785-91.
28. Autodock Forum. ADL, Parameters for docking with metal ions in receptor, 2009 (accessed November 2016), http://autodock.1369657.n2.nabble.com/ADL-Parameters-for-docking-with-metal-ions-in-receptor-td2505649.html.
29. Arce ER, Mosquillo MF, Pérez-Díaz L, Echeverría GA, Piro OE, Merlino A, Coitiño EL, Ribeiro CM, Leite CQ, Pavan FR, Otero L. Aromatic amine N-oxide organometallic compounds: searching for prospective agents against infectious diseases. Dalton Trans, 2015; 44(32): 14453-64.
30. Molegro Molecular Viewer, version 2.5. Molegro -a CLC bio company; Aarhus, Denmark: 2012.
31. Owolabi J, Omogbai EKI, Obasuyi O. Antifungal and antibacterial activities of the ethanolic and aqueous extract of *Kigelia africana* (Bignoniaceae) stem bark. Afr J Biotechnol, 2007; 6(14): 882–85.
32. Bhattacharya K, Samanta SK, Tripathi R, Mallick A, Chandra S. Apoptotic effects of mahanine on human leukemic cells are mediated through crosstalk between Apo 1/Fas signaling and the Bid protein and via mitochondrial pathways. Biochem Pharmacol, 2010; 79(3): 361–72.
33. Alakurtti S, Makela T, Koskimies S, Yli-Kauhaluoma J. Pharmacological properties of the ubiquitous natural product betulin. Eur J Pharm Sci, 2006; 29(1): 1-13.
34. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol, 2007; 35(4): 495- 516.
35. Willmann M, Wacheck V, Buckley J, Nagy K, Thalhammer J, Paschke, R, Triche, T, Jansen B, Selzer E. Characterization of NVX-207, a novel betulinic acid-derived anticancer compound. Eur J Clin Invest, 2009; 39(5): 384- 94.
36. Martin SJ, Diego S. Apoptosis and cancer. In: Seamus JM (Ed). Apoptosis and Cancer: An Overview, 1997; 1–22 (New York: Karger Landes Systems).
37. Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem, 1997; 45(7): 923–34.
38. Liu MC, Yang SJ, Jin LH, Hu DY, Xue W, Song BA, Yang S. Synthesis and cytotoxicity of novel ursolic acid derivatives containing an acyl piperazine moiety. Eur J Med Chem, 2012; 58: 128–35.
39. Guha M, Maity P, Choubey V, Mitra K, Reiter RJ, Bandyopadhyay U. Melatonin inhibits free radical mediated mitochondrial-dependent hepatocyte apoptosis and liver damage induced during malarial infection. J Pineal Res, 2007; 43(4): 372–81.
40. Zhou HB, Zhu JR. Paclitaxel induces apoptosis in human gastric carcinoma cells. World J Gastroentero, 2003; 9(3): 442–5.
41. Mills KR, Reginato M, Debnath J, Queenan B, Brugge JS. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation *in vitro*. Proc Natl Acad Sci U S A, 2004; 101(10): 3438–43.
42. Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res, 2005; 65: 613-21.
43. Lopez-lazaro, M. Digitoxin as an anticancer agent with selectivity for cancer cells: possible mechanisms involved. Expert opin ther targets, 2007; 11(8): 1043-53.
44. Bui T, Thompson CB. Cancer's sweet tooth. Cancer cell, 2006; 9: 419-20.
45. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. Cancer Cell, 2006; 9: 425–34.
46. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci U S A, 2010; 107(5): 2037–42.

47. Xie H, Valera VA, Merino MJ, Amato AM, Signoretti S, Linehan WM, Sukhatme VP, Seth P. LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. Mol Cancer Ther, 2009; 8(3): 626–35.
48. Sheng SL, Liu JJ, Dai YH, Sun XG, Xiong XP, Huang G. Knockdown of lactate dehydrogenase A suppresses tumor growth and metastasis of human hepatocellular carcinoma. FEBS J, 2012; 279: 3898–910.