Indian Journal of Experimental Biology
Vol. 51, July 2013, pp. 485-491

Anticancer activity of betulinic acid on MCF-7 tumors in nude mice

Archana A Damle*, Yogita P Pawar & Archana A Narkar

Radiation Medicine Centre (BARC), Tata Memorial Hospital Annexe,
Jerbai Wadia Road, Parel, Mumbai 400 012, India

Received 5 February 2013; revised 15 April 2013

Breast cancer is a major public health problem and the low effectiveness of conventional therapies to achieve long term survival results in increased mortality associated with advanced breast cancers. Betulinic acid (BA) is a pentacyclic triterpene which can be isolated from number of plants grown in the tropics. It exhibits cytotoxic activity against variety of cancer cell lines. In the present study, the *in vitro* cytotoxic activity and *in vivo* antitumor activity of BA was evaluated in athymic nude mice bearing MCF-7 breast adenocarcinoma xenografts. *In vitro* cytotoxic activity of BA on MCF-7 cells was studied using the MTT assay and BA was cytotoxic towards MCF-7 cells with IC_{50} value of 13.5µg/mL. The antitumor activity of BA was studied at concentrations of 50 and 100 mg/kg body weight in mice injected with MCF-7 cells. BA treatment delayed tumor formation and statistically significant reduction (P<0.0001) of 52 and 77% in the tumor size at concentrations of 50 and 100 mg, respectively was observed. Histopathological analysis of tumors revealed decreased angiogenesis, proliferation and invasion in BA treated animals. This is one of the first studies demonstrating the *in vivo* antitumor activity of BA on MCF-7 breast cancer tumors in nude mice. The antitumor effect of BA can further be enhanced by use of combination therapy and novel drug delivery systems, thus making it a promising candidate for management of breast cancer patients.

Keywords: Betulinic acid, Breast cancer, MCF-7, Nude mice

The evaluation of potential antitumor activity of approximately 2500 extracts derived from globally collected plants resulted in isolation and identification of betulinic acid (BA) as a melanoma specific cytotoxic agent belonging to the class of pentacyclic lupane type triterpenoids[1]. Betulinic acid (3β, 3-hydroxy-lup-20(29)-en-28-oic acid) structure consist of a 30 carbon skeleton having molecular formula $C_{30}H_{48}O_3$ and has three available sites for simple chemical modifications at C-3, C-20 and C-28 (Fig.1). It is a highly lipophilic molecule with limited water solubility. Betulinic acid can be directly isolated from various plants widespread in the tropics. It can also be chemically derived from betulin, a substance found in abundance in the outer bark of white birch trees. Though initial reports suggested that BA is selectively cytotoxic to melanoma cells[1], it was subsequently demonstrated that it exhibited several medicinal and biological activities including antibacterial, antimalarial, and antiHIV[2-4]. The *in vitro* antitumor cytotoxic activity of BA has been illustrated in broad spectrum of cancer cell lines including those of leukemia, neuroblastoma, colon, breast, melanoma, lung, prostrate, and cervical origin[1,5-10]. Breast cancer is a major public health problem affecting millions of women globally. It is responsible for an estimated 16% and 12% of all cancer deaths in women in developed and developing countries, respectively[11]. In India, approximately 75-80,000 new cases are diagnosed annually and in developing countries, including India, 30-70% of the patients are diagnosed with locally advanced disease[12-13]. The low effectiveness of conventional therapies to achieve long term survival results in increased mortality associated with advanced breast cancers. Hence, new therapies which use novel mechanisms to induce tumor cell death are need of the hour. It has been reported that BA cytotoxicity in cancer cells is via the intrinsic pathway involving triggering of mitochondria mediated apoptosis via direct mitochondrial perturbations. Perturbance of

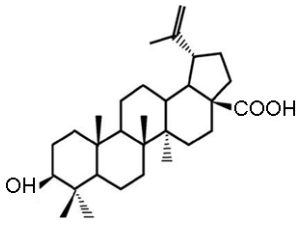

Fig. 1—Chemical structure of Betulinic acid.

Correspondent author
Telphone: +91-22-2413 4960/+91-22-2414 9428
Fax: +91-22- 24157098
E-mail: archanadamle10@gmail.com

mitochondrial function constitutes a central coordinating event in BA induced apoptosis involving cytochrome c release, caspase activation and apoptotic DNA fragmentation[1]. Proteins of Bcl-2 family which comprise both anti-apoptotic eg. Bcl-2, Bcl-X_L, Mcl-1, as well as pro-apoptotic molecules such as Bax, Bak, Bad and BH3 domain only molecules are key signal transduction members involved in BA induced apoptosis. Also, generation of reactive oxygen species upon BA treatment has been reported to be responsible in initiating mitochondrial membrane permeability and incubation with antioxidants rescue cells from undergoing apoptosis[8-10,14-16]. Thus, mitochondrion-targeted agents such as BA may open new perspective in management of cancers refractory to conventional therapies. The present study explores the use of betulinic acid as an *in vivo* antitumor agent in an animal model of human breast cancer.

Materials and Methods

In vitro antiproliferative activity of betulinic acid—Human breast adenocarcinoma cell line, MCF-7 was obtained from National Centre for Cell Science, Pune, India. Betulinic acid (BA), DMSO and MTT (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemicals, USA. All the other reagents used were of highest quality and purchased locally. MCF-7 cells were grown in Iscove's modified Dulbecos medium (IMDM, Gibco-BRL) with 10% fetal bovine serum (FBS, Biological Industries, Israel) at 37 °C in a humidified atmosphere of 5% CO_2. Exponentially growing cells were seeded in 96 well tissue culture plates at a density of 2×10^4 cells per well and incubated for 24 h before treatment. BA was dissolved in DMSO at a concentration of 5 mg/mL, diluted with IMDM with FBS and added to MCF-7 cells, in triplicate wells, at an increasing concentration of 5-20 µg/mL. The cultures were incubated at various time intervals and the antiproliferative activity of BA was tested using the MTT assay as described earlier[17]. Briefly, at the end of the respective BA treatment, MTT (5 mg/mL) was added to the wells and the plates were incubated at 37 °C for 3 h. The formazan crystals were dissolved with 10% SDS overnight and optical density was measured at 540 nm with a reference of 690 nm. Concentration that inhibited cell growth by 50% (IC_{50}) as compared to the non-treated cells at 48 h was determined.

In vivo antitumor activity of BA in nude mice—Female Balb/c athymic nude mice were purchased from National Institute of Virology, Pune and were cared for and used in accordance with institutional guidelines. Since these mice are athymic, they lack the ability to reject human grafts. The experimental model used in the present study involved MCF-7 breast adenocarcinoma cell line which is of human origin. Hence, nude mice were used to transplant these cells as they will grow and form solid tumors which can be used to study the antitumor effect of BA. All the animals were housed in isolators with filter top cages and maintained in sterile environment with daily 12 h light/12h dark cycle, 50% RH and 20-22 °C temperature. MCF-7 cells were grown in tissue culture flasks and harvested using Trypsin-EDTA (Hi Media, India). The cell pellets were suspended in IMDM and 1×10^6 cells were injected sub-cutaneously in right flanks of the mice. BA was injected on the day following the injection of MCF-7 cells. Betulinic acid was dissolved in DMSO and two doses of 50 and 100 mg/kg body weight were given intraperitonealy to check the antitumor activity *in vivo*. A total of 6 doses of BA were given at 3-4 days interval. Mice were divided in the following four groups: Group 1 (Control): mice injected with MCF-7 cells alone, Group 2 (Vehicle control): mice injected with MCF-7 and DMSO, Group 3 (BA treated): mice injected with MCF-7 and 50 mg BA and Group 4 (BA treated): mice injected with MCF-7 and 100 mg BA. Each group comprised of minimum of 5-6 animals. The experiment was repeated twice. The basic protocol was similar to that reported by Pisha *et al*[1]. The tumor size was measured using vernier callipers and readings were taken of four different dimensions for each tumor. The tumors were measured at regular intervals up to 35-40 days post injection of MCF-7 cells. The project was approved by institutional animal ethics committee and the experiments were carried out as per the guidelines.

Histopatological studies—The mice were dissected and representative tumors from all four groups were collected in 10% neutral buffered formalin. The tumors were embedded in paraffin blocks, 1-2 µm thick sections were taken and routine Haematoxylin-Eosin staining was carried out to observe and compare the histological characteristics. Various parameters like tumor proliferation, mitotic index and angiogenesis were studied and compared in each group of animals.

Statistical analysis—Student's *t*-test was used to compare the tumor sizes and the mitotic index of control and BA treated mice.

Results

In vitro cytotoxic activity—The cytotoxic activity of betulinic acid on MCF-7 cell line was determined *in vitro.* Betulinic acid was insoluble in aqueous solutions hence it was dissolved in DMSO, diluted with culture medium and added to MCF-7 cells in triplicates. The concentrations of BA used were between 0-20 µg/mL. As illustrated in Fig.2, betulinic acid exerted time and concentration dependant decrease on the viability of MCF-7 cells as estimated by MTT assay. The viability of cells decreased with increasing concentrations of BA and the IC_{50} of BA for MCF-7 cells at 48 h was calculated to be 13.5 µg/mL.

In vivo antitumor activity of BA—The *in vivo* antitumor activity of BA was determined in an animal model transplanted with MCF-7 human breast adenocarcinoma cells. The cells were cultured *in vitro*, trypsinized and injected subcutaneously in nude mice. Two concentrations of BA (50 and 100 mg) were used and a total of six doses of each concentration were administered intraperitoneally in mice to check the antitumor activity of BA. Tumors were visible at 10-15 days post injection of MCF-7 cells. The tumor dimensions were measured at various time intervals up to five weeks in each mouse. Measurable tumors were seen from 13th day after transplanting MCF-7 cells in control and DMSO injected mice (Fig. 3). In mice treated with 50 and 100 mg of BA, the tumor formation was delayed with visible tumors seen from 21st and 25th day, respectively, indicating that BA inhibited early tumor formation in these mice. The mean tumor volume at five weeks in the different groups was as follows: control (1.33 ± 0.06), vehicle control (1.27 ± 0.09), BA treated group I (0.64 ± 0.06) and BA treated group II (0.31 ± 0.07). Thus, it is clear that BA exhibits a potent antitumor effect on MCF-7 tumors with 52 and 77% reduction in the tumor size at concentrations of 50 and 100 mg, respectively at 37 days post injection. Also, this reduction was statistically significant (P<0.0001) as compared to the control mice. It has to be noted that similar reduction in the tumor dimensions was seen at all the time points in both groups of BA treated animals (50 and 100 mg) as compared to the control mice with $P < 0.0001$ for each individual point. No significant difference ($P > 0.05$) was observed between the tumor size in vehicle control and control animals at any point of time indicating that DMSO per se does not have any effect on tumor growth. None of the BA treated mice showed any signs of toxicity or body weight loss as compared to control animals.

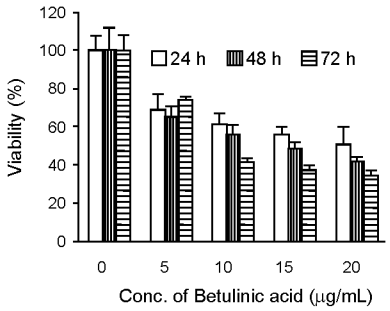

Fig. 2—*In vitro* cytotoxic effect of betulinic acid on MCF-7 cells.

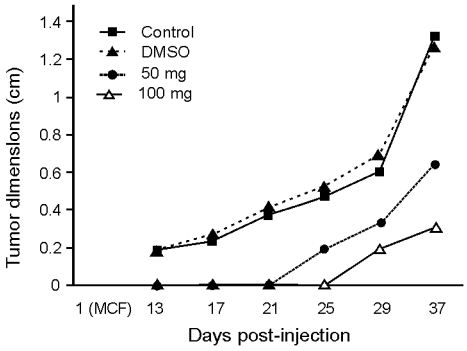

Fig. 3—*In vivo* antitumor activity of betulinic acid in nude mice xenografted with MCF-7 cells.

Histopathological analysis of tumors—Representative tumors from all four groups were collected and processed as per standard histopathological protocol. Various microscopic features like angiogenesis, invasiveness and mitotic index were studied on Haematoxylin-Eosin stained tumor sections. Formation of increased number of new blood vessels depicting angiogenesis was observed in tumor sections from control mice (Fig. 4a) as compared to animals treated with 100 mg of BA (Fig. 4b). The microscopic examination of tumor sections revealed that tumor proliferation and invasion is much more evident in control group (Figs. 5a and 6a) as that observed in BA treated group (Figs. 5b and 6b). The overall comparison of tumor size and the histological parameters of the tumors from control and BA treated group are given in Table 1. The overall tumor dimensions were significantly less in BA treated mice. A significant decrease in the mitotic index was also

observed in mice treated with 50 and 100 mg of BA ($P< 0.0001$).

Discussion

The present report describes the cytotoxic activity of betulinic acid on MCF-7 cells *in vitro* and antitumor activity in nude mice xenografted with MCF-7 breast adenocarcinoma cells. Betulinic acid was first purified and identified to selectively inhibit the growth of human melanoma in cell cultures and animal models[1]. However, subsequent studies showed that cytotoxicity of BA is not limited to melanoma

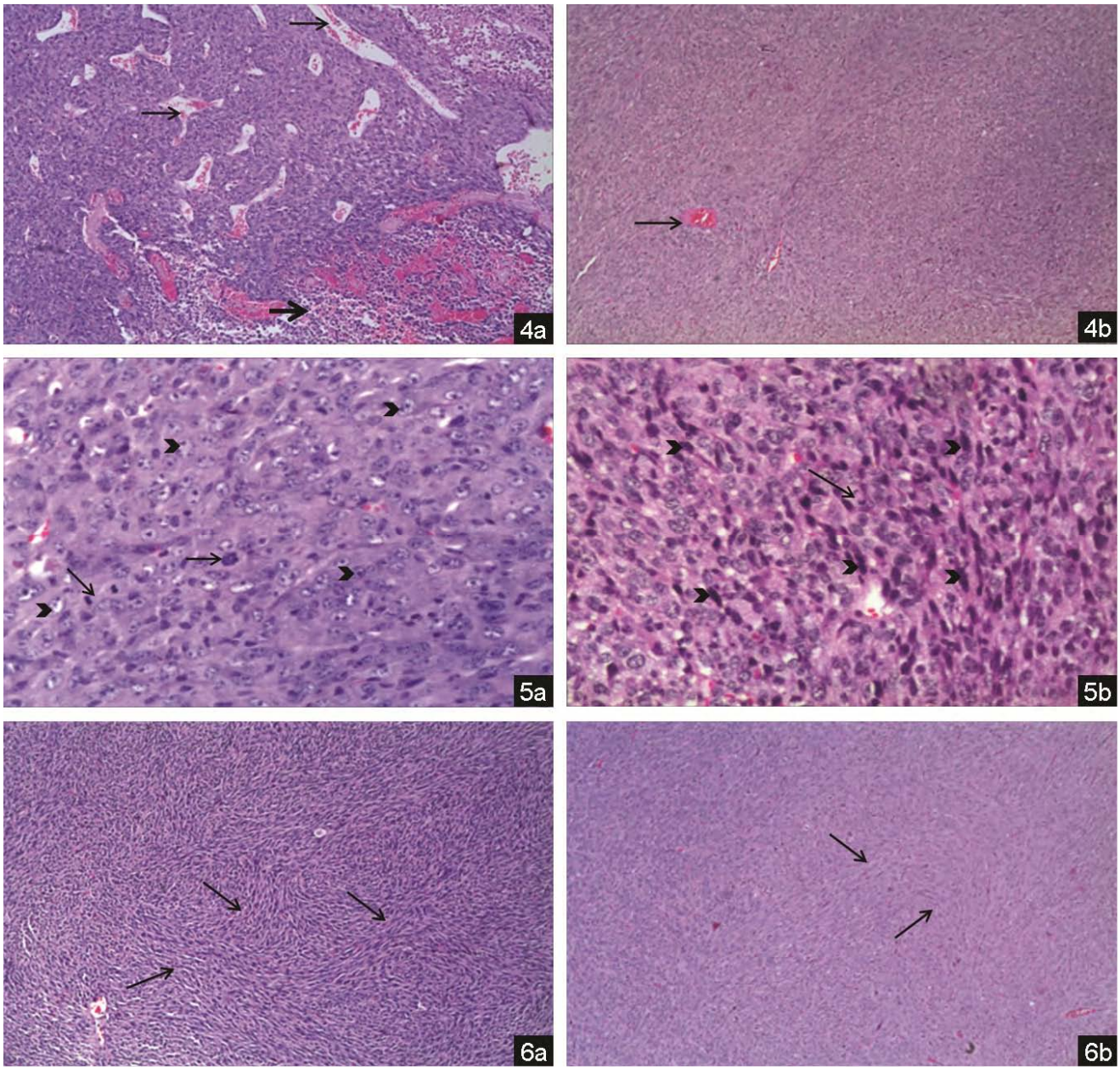

Fig. 4—Tumor section from control mice xenografted with MCF-7 cells. Multifocal area depicting significant angiogenesis (thin arrow) and area of necrosis with hemorrhage (thick arrow) (5x). (b): Tumor section from BA (100 mg) treated mice xenografted with MCF-7 cells. Multifocal area showing minimal angiogenesis (thin arrow) (5x). Fig. 5—(a): Tumor section from control mice xenografted with MCF-7 cells. Pleomorphic neoplastic cells (arrow head) with variable mitotic figures (thin arrow) and hyperchromatic nucleus indicative of cell proliferation (40×). (b): Tumor section from BA (100 mg) treated mice xenografted with MCF-7 cells. Pleomorhphic cells with minimal mitotic activity (thin arrow) and condensed cell nucleus (arrow head) suggestive of apoptosis (40x). Fig. 6—(a): Tumor section from control mice xenografted with MCF-7 cells. Proliferating cells with haphazardly arranged invasive cells (thin arrow) (5x). (b): Tumor section from BA (100 mg) treated mice xenografted with MCF-7 cells. Proliferating cells with minimal invasion (thin arrow) (5x).

Table 1—Comparison of tumor size and various histological characteristics of tumors from control and BA treated group. (The tumor dimensions are 5 weeks post injection)

[Values are mean ± SD from 10 observation each]

	Group1 (Control)	Group 2 (Vehicle control)	Group 3 (BA-50 mg)	Group 4 (BA-100 mg)
Tumor size	1.33 ± 0.06^{a}	1.27 ± 0.09^{b}	0.64 ± 0.06^{c}	0.31 ± 0.07^{d}
Angiogenesis	Multifocal (2+)	Multifocal (2+)	Multifocal (2+)	Multifocal (1+)
Invasion	3+	3+	3+	1+
Mitotic index	0.13 ± 0.02^{e}	0.12 ± 0.06^{f}	0.07 ± 0.01^{g}	0.05 ± 0.02^{h}

a – b: $P = 0.09$, a – c: $P < 0.001$, a – d: $P < 0.001$
e – f : $P = 0.62$, e – g : $P < 0.001$, e – h: $P < 0.001$
Student's *t* test

cells and it acts on variety of cancer cell lines *in vitro*[1,5-10,15]. In the present study, BA being a lipophilic compound was dissolved in DMSO and evaluated for its cytotoxic activity against MCF-7 cells. BA exhibited a time and dose dependant cytotoxic effect on MCF-7 cell lines with IC_{50} value of 13.5 µg/mL. Similar IC_{50} values (10-15 µg/mL) against various cancer cell lines including MCF-7 have been reported with betulin which is another most studied triterpene of plant origin[16]. Several extracts of a native plant of Morroco exhibited potent cell proliferation inhibition on MCF-7 cells, out of which the ethylacetate extract containing BA was found to be most active with IC_{50} value of 279.51±16.0 µg/mL[18]. Though, this IC_{50} value is much higher (may be due to the crude nature of the extract) as compared to that obtained in the present study, it corroborates the finding emphasizing the cytoxicity of BA on MCF-7 cells. IC_{50} values of 6-14 µM (MCF-7: 12.2 µM) have been reported with BA on a panel of tumor cell lines and the carbamate derivatives of BA and betulin show selective toxicity against target cell lines with reduced toxic effect on normal human fibroblast. The mechanism of cell death in these studies is by induction of apoptosis[15]. Comparison of the antiproliferative activity of BA with doxorubicin revealed that BA cytotoxicity was independent of tumor type and p53 status. Moreover, in spite of the lower potency as compared to doxorubicin, BA seemed to be more selective for tumor cells exhibiting 2-5 and 1000 fold less toxicity towards normal human fibroblast and peripheral blood leucocytes respectively as compared to doxorubicin[10]. The non toxicity of BA has been emphasized in the present study as well as earlier studies[1,15]. A number of novel semi-synthetic derivatives of BA and betulin were screened for *in vitro* cytotoxic activity on various human cancer cell lines. The percentage proliferation indicated that the different compounds were more potent against Hela, HepG2, and Jurkat cell lines whereas not much difference was observed on other cell lines including MCF-7[19]. The 3-*O*-acylated derivatives of BA showed IC_{50} <10 µg/mL against human lung carcinoma cell line (A549) which was better than BA. In an ovarian cancer cell line, all derivatives prepared showed weaker cytotoxicity than BA[20].

As mentioned above, the *in vitro* cytotoxic activity of BA on tumor cells of diverse histological origin has been demonstrated. However, there are few reports on the *in vivo* antitumor activity of BA. In one of the earliest study conducted in athymic mice carrying human melanoma, it was shown that tumor growth was completely inhibited without any toxicity and the antitumor activity of BA was mediated by the induction of apoptosis[1]. In the present study, the effect of BA on MCF-7 human breast adenocarcinoma induced tumors has been demonstrated in athymic nude mice. In the present experiments, besides the cytotoxic activity on MCF-7 cancer cells *in vitro*, betulinic acid also effectively suppressed growth of MCF- 7 tumors in an animal model of human breast carcinoma by delaying the development of MCF-7 tumors in a dose dependant manner. Also, significant reduction in the tumor volume was observed in mice treated with BA as compared to control animals and no signs of acute or chronic toxicity were observed in any of these mice. Mertens-Talcott *et al.*[21] reported that BA decreases ER-negative breast cancer cell growth *in vitro* and *in vivo* and this effect is at least in part based on interaction with the microRNA-27a-ZBTB10SP-axis.

In vivo antitumor activity of BA has been demonstrated in animal models of ovarian

carcinoma[10], colon cancer[6] and rhabdomyosarcoma[14]. BA also inhibited tumor formation in mouse skin two stage carcinogenesis[22]. These mice did not show any sign of apparent toxicity or body weight loss as compared with controls. To increase the effectiveness of BA as anticancer agent, BA derivative were tried and they exhibited favourable pharmacokinetic characteristics of a systemically administered drug and showed better *in vivo* antitumor efficacy as compared to BA in a human colon cancer xenograft model[23]. The weak hydro-solubility of BA makes it difficult to study its *in vivo* efficacy and a pharmaceutical formulation is not yet available, hence liposome formulation of BA has been developed and reported to have significant *in vivo* antitumor activity on human colon and lung cancer xenografts in nude mice[24]. The *in vivo* efficacy of BA and its derivatives in animal models has been compared individually with other anticancer agents like cyclophosphamide, 5-flurourasil on liver cancer[25] or in combination with mithramycin for pancreatic cancer[26] and vincristine for suppression of lung metastasis[27]. Lack of toxicity together with significant antitumor activity leads to a favourable therapeutic index which is a prerequisite for any drug to be considered for development. This criterion is fulfilled by BA, as shown in the present and the earlier reports on the anticancer activities of BA[1,6,10,24].

In the present studies, tumor sections from BA treated animal revealed decrease in the mitotic index and the cells exhibited reduced invasive potential as compared to the tissues from control mice. Decreased angiogenesis and presence of apoptotic cells was also evident in tumor section from BA treated mice. Number of other studies have also attributed the cytotoxic activity of BA and its derivatives to their ability to induce apoptosis and inhibit angiogenesis *in vitro* as well as in experimental tumor models[8,10,14-16,27-29]. Specificity protein (Sp) transcriptional factors play a role in BA induced antitumor activity by inhibiting cancer cell growth[6,21,26]. BA causes cell death through induction of apoptosis in leukemia[30] and Ewings sarcoma cell line[9]. Also, BA induced apoptosis in breast cancer cell lines by activating mitochondrial pathway[31]. This finding along with the presence of apoptotic cells in the tumor sections from BA treated mice are suggestive of the fact that the possible mechanism of action of BA on MCF-7 breast cancer tumors in nude mice could be through mitochondria mediated apoptosis.

Conclusions

The antitumor activity of betulinic acid in an *in vivo* model of MCF-7 cells induced breast adenocarcinoma has been demonstrated. Treatment with BA delayed tumor formation, decreased cell proliferation, invasion and angiogenesis in these mice. These findings along with lack of systemic toxicity emphasize the importance of BA as a promising candidate for treatment of breast carcinoma. Use of combination therapy and novel drug delivery systems will be helpful to further enhance the antitumor activity of BA, thus making it a potential option for management of breast cancer patients.

References

1 Pisha E, Chai H, Lee I S, Chagwedera T E, Farnsworth N R, Cordell G A, Beecher C W, Fong H H S, Kinghom A D, Brown D M, Wani M C, Wall M E, Hieken T J, Gupta T K D & Pezzuto J M, Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis, *Nat Med*, 1 (1995) 1046.

2 Bringmann G, Saeb W, Assi L A, François G, Sankara Narayanan A S, Peters K & Peters E M, Betulinic acid: isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, antimalarial acticvity, and crystal structure of the benzyl ester, *Planta Med*, 63 (1997) 225.

3 Cichewicz R H & Kouzi S A, Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection, *Med Res Rev*, 24 (2004) 90.

4 Yogeeswari P & Sriram D, Betulinic acid and its derivatives: a review on their biological properties, *Curr Med Chem*, 12 (2005) 657.

5 Chintharlapalli S, Papineni S, Ramaiah S K & Safe S, Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors, *Cancer Res*, 67 (2007) 2816.

6 Chintharlapalli S, Papineni S, Lei P, Pathi S & Safe S, Betulinic acid inhibits colon cancer cell and tumor growth and induces proteasome dependent and -independent downregulation of specificity proteins (Sp) transcription factors, *BMC Cancer*, 11 (2011) 371.

7 Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer P H, Peter M E & Debatin K M, Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors, *Cancer Res*, 57 (1997) 4956.

8 Fulda S & Kroemer G, Targeting mitochondrial apoptosis by betulinic acid in human cancers in human cancers, *Drug Discovery Today*, 14 (2009) 885.

9 Raghuvar Gopal D V, Narkar A A, Badrinath Y, Mishra K P & Joshi D S, Betulinic acid induces apoptosis in human chronic myelogenous leukemia (CML) cell line K-562 without altering the levels of Bcr-Abl, *Toxicol Lett*, 15 (2005) 343.

10 Zuco V, Supino R, Righetti, S C, Cleris L, Marchesi E, Gambacorti-Passerini C & Formelli F, Selective cytotoxicity

of betulinic acid on tumor cell lines, but not on normal cells, *Cancer Lett*, 175 (2002) 17.

11 Mittra I, Mishra G A, Singh S, Aranke S, Notani P, Badwe R, Miller A B, Daniel E E, Gupta S, Uplap P, Thakur M H, Ramani S, Kerkar R, Ganesh B & Shastri S S, A cluster randomized, controlled trial of breast and cervix cancer screening in Mumbai, India: Methodology and interim results after three rounds of screening, *Int J Cancer*, 15 (2010) 976.

12 Chopra R, The Indian scene, *J Clin Oncol*, 19 (2001) 106S.

13 Valero V V, Buzdar A U & Hortobagyi G N, Locally advanced breast cancer, *Oncologist*, 1 (1996) 8.

14 Eichenmüller M, Hemmerlein B, von Schweinitz D & Kappler R, Betulinic acid induces apoptosis and inhibits hedgehog signalling in rhabdomyosarcoma, *Br J Cancer*, 29 (2010) 43.

15 Kommera H, Kalud-erovic G N, Dittrich S, Kalbitz J, Dräger B, Mueller T & Paschke R, Carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity, *Bioorg Med Chem Lett*, 20 (2010) 3409.

16 Li Y, He K, Huang Y, Zheng D, Gao C, Cui L & Jin Y H, Betulin induces mitochondrial cytochrome c release associated apoptosis in human cancer cells, *Mol Carcinog*, 49 (2010) 630.

17 Kano Y, Akutsu M, Tsunoda S, Mano H, Sato Y, Honma Y Furukawa Y, In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agent, *Blood*, 97 (2001) 1999.

18 Chaouki W, Leger D Y, Eljastimi J, Beneytout J L & Hmamouchi M, Antiproliferative effect of extracts from *Aristolochia baetica* and *Origanum compactum* on human breast cancer cell line MCF-7, *Pharm Biol*, 48 (2010) 269.

19 Santos R C, Salvador J A, Marín S, Cascante M, Moreira J N & Dinis T C, Synthesis and structure-activity relationship study of novel cytotoxic carbamate and N-acylheterocyclic bearing derivatives of betulin and betulinic acid, *Bioorg Med Chem*, 15 (2010) 4385.

20 Ahmad F B H, Ghaffari Moghaddam M G, Basri M & Abdul Rahman M B, Anticancer activity of 3-O-acylated betulinic acid derivatives obtained by enzymatic synthesis, *Biosci Biotechnol Biochem*, 74 (2010) 1025.

21 Mertens-Talcott S U, Noratto G D, Li X, Angel-Morales G, Bertoldi M C & Safe S, Betulinic acid decreases ER-negative breast cancer cell growth *in vitro* and *in vivo*: Role of Sp transcription factors and microRNA-27a:ZBTB10, *Mol Carcinog*,Mar 7 (2012) doi: 10.1002/mc.21893.

22 Yasukawa K, Takido M, Matsumoto T, Takeuchi M & Nakagawa S, Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis, *Oncology*, 48 (1991) 72.

23 Rajendran P, Jaggi M, Singh M K, Mukherjee R & Burman A C, Pharmacological evaluation of C-3 modified betulinic acid derivatives with potent anticancer activity, *Invest New Drugs*, 26 (2008) 25.

24 Mullauer F B, van Bloois L, Daalhuisen J B, Ten Brink M S, Storm G, Medema J P, Schiffelers R M & Kessler J H, Betulinic acid delivered in liposomes reduces growth of human lung and colon cancers in mice without causing systemic toxicity, *Anticancer Drugs,* 22 (2011) 223.

25 Bi Y, Xu J, Wu X, Ye W, Yuan S & Zhang L, Synthesis and cytotoxic activity of 17-carboxylic acid modified 23-hydroxy betulinic acid ester derivatives, *Bioorg Med Chem Lett*, 17 (2007) 1475.

26 Gao Y, Jia Z, Kong X, Li Q, Chang D Z, Wei D, Le X, Huang S, Wang L, Xie K, Combining betulinic acid and mithramycin A effectively suppresses pancreatic cancer by inhibiting proliferation, invasion and angiogenesis, *Cancer Re*s, 71 (2011) 5182.

27 Sawada N, Kataoka K, Kondo K, Arimochi H, Fujino H, Takahashi Y, Miyoshi T, Kuwahara T, Monden Y, Ohnishi Y, Betulinic acid augments the inhibitory effects of vincristine on growth and lung metastasis of B16F10 melanoma cells in mice, *Br J Cancer*, 90 (2004) 1672.

28 Karna E, Szoka L & Palka J A, Betulinic acid inhibits the expression of hypoxia inducible factor 1-alpha and vascular endothelial growth factor in human endometrial adenocarcinoma cells, *Mol Cell Biochem*, 340 (2010) 15.

29 Kwon H J, Shim J S, Kim J H, Cho H Y, Yum Y N, Kim S H & Yu J, Betulinic acid inhibits growth factor-induced *in vitro* angiogenesis via the modulation of mitochondrial function in endothelial cells, *Jpn J Cancer Res*, 93 (2002) 417.

30 Raghuvar Gopal D V, Narkar A A, Badrinath Y, Mishra K P & Joshi D S, Protection of Ewing's sarcoma family tumor (ESFT) cell line SK-N-MC from betulinic acid induced apoptosis by alpha DL-tocopherol, *Toxicol Lett*, 153 (2004) 201.

31 Raghuvar Gopal D V, Studies on the anticancer spectrum and mechanisms of betulinic acid induced apoptosis in human cancer cell lines, *Ph.D thesis*, University of Mumbai (2005), 89.