BETULINIC ACID WAS MORE CYTOTOXIC TOWARDS THE HUMAN BREAST CANCER CELL LINE MDA-MB-231 THAN THE HUMAN PROMYELOCYTIC LEUKAEMIA CELL LINE HL-60

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Betulinic acid (BA) is a pentacyclic triterpene found in several botanical sources that has been shown to cause apoptosis in a number of cell lines. This study was undertaken to determine the in vitro cytotoxic properties of BA towards the human mammary carcinoma cell line MDA-MB-231 and the human promyelocytic leukaemia cell line HL-60 and the mode of the induced cell death. The cytotoxicity and mode of cell death of BA were determined using the MTT assay and DNA fragmentation analysis, respectively. In our study, the compound was found to be cytotoxic to MDA-MB-231 and HL-60 cells with IC50 values of 58 μg/mL and 134 μg/mL, respectively. Cells treated with high concentrations of BA exhibited features characteristic of apoptosis such as blebbing, shrinking and a number of small cytoplasm body masses when viewed under an inverted light microscope after 24 h. The incidence of apoptosis in MDA-MB-231 was further confirmed by the DNA fragmentation analysis, with the formation of DNA fragments of oligonucleosomal size (180-200 base pairs), giving a ladder-like pattern on agarose gel electrophoresis. BA was more cytotoxic towards MDA-MB-231 than HL-60 cells, and induced apoptosis in MDA-MB-231 cells.

Keywords: Betulinic Acid, Cytotoxic, Apoptosis

INTRODUCTION

Betulinic acid [3β-hydroxy-lup-20(19) lupaen-28-carboxylic acid] (BA) is a pentacyclic triterpene (Fig. 1). It occurs in several botanical sources and is found in abundance in the outer bark of white birch trees, Betula alba L. (Pisha et al. 1995). BA has a molecular weight of 456.70 and the molecular chemical formula is C30H48O3. It appears as a white to off-white powder, and is highly solubilised in dimethyl sulphoxide.

BA has been known to induce apoptosis selectively in human melanoma cells (Pisha et al. 1995; Wick et al. 1999; Fulda and Debatin 2001) and also to inhibit the growth of tumour cells of neuroectodermal
origin (Schmidt et al. 1997; Fulda et al. 1999). It has been found that the anti tumour activity of BA was not only restricted to melanoma but was also active on non-melanoma tumours after it has been shown to have significant in vivo antitumour activity on ovarian carcinoma IGROV-1 xenografts. In fact, it was discovered that BA has cytotoxic effects on several different types of cell lines including melanomas, small- and non-small cell lung carcinomas, and ovarian and cervical carcinomas (Zuco et al. 2002). The ability to selectively induce apoptosis suggests that BA might be a potent candidate for future cancer chemotherapy (Hata et al. 2003).

Additionally, it has been suggested that BA is more applicable for management of cancer than some anti-tumour drugs. It not only inhibited growth and induced apoptosis in human leukaemic cell line K562, but was also very effective in blocking the proliferation of Lucena 1, a vincristine-resistant derivative of K562 that displays several multidrug resistance (MDR) characteristics (Fernandes et al. 2003). It has also been reported that BA is cytotoxic to small lung cancer doxorubicin-resistant cells (POGB/DX) and the small cell lung carcinoma (POGB) to some extent. Despite having lower potency when compared with doxorubicin, BA seems to be selective for tumour cells (Zuco et al. 2002).

BA has also been found to retard the progression of HIV infection, (Hashimoto et al. 1997; Vlietinck et al. 1998; Smith et al. 2001), and it has been reported to have anti-malarial (Hanne et al. 2004) and anti-inflammatory effects (Recio et al. 1995; Mukherjee et al. 1997).

The objective of this study was to determine the in vitro cytotoxic properties of BA towards the human mammary carcinoma (MDA-MB-231) and the human promyelocytic leukaemia (HL-60) cell lines, and the mode of cell death of following exposure to BA.

METHODS

Compound

Betulinic acid (purity of ~97%) was kindly supplied by Professor Dr. Faujan Haji Ahmad from the Department of Chemistry, Faculty of Sciences, Universiti Putra Malaysia. The compound was dissolved in dimethylsulphoxide (DMSO) to give a stock concentration of 10 mg/mL.
Cells

The human mammary carcinoma cell line MDA-MB-231 and the human promyelocytic leukaemia cell line HL-60 were purchased from the American Type of Culture Collection (ATCC), USA. The cells were grown in RPMI 1640 medium supplemented with 10% of foetal calf serum and antibiotics (100 IU of penicillin/100 mg/mL of streptomycin). The cells were maintained in a 25 cm² T-flask and incubated at 37°C under 5% CO₂ in a humidified atmosphere. The cell viability was measured by staining the cells with trypan blue and counted in a haemocytometer under a light microscope.

Determination of Cytotoxicity

The cytotoxicity assay was performed in a 96-well microtitration plate. The MDA-MB-231 and HL-60 cells at an initial concentration of 1 x 10⁶ cells/mL were treated with 140, 120, 100, 80, 60, 40 and 20 µg/mL of BA. Initially, various concentrations of the compound were prepared from the sub-stock solution by serial dilution in RPMI 1640 to give a volume of 100 µL in each of the microtitre plate wells (Shier 1999). Subsequently, 100 µL of the cell suspension was added into each well. A control well without the compound was included. The assay was performed in triplicates. The plate was then incubated at 37°C, 5% CO₂ and 90% humidity for 72 h.
MTT Assay

The cytotoxicity of BA was quantitatively estimated by a non-radioactive, colorimetric assay system using a tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil-tetrazolium bromide (MTT) (Mosman 1983). Briefly, MTT was dissolved in phosphate-buffered saline at 5 mg/mL and filter-sterilised to remove the small amount of insoluble residue present in some batches. The MTT solution was then added directly to all appropriate microtitre plate wells (10 µL per 100 µL medium) containing cells and complete growth medium, with or without the tested compound. The plate was then incubated for 4 h at 37ºC to allow MTT to metabolise to formazan. Subsequently, the supernatant was aspirated and 100 µL of DMSO was added and mixed thoroughly to dissolve the dark blue formazan crystals. The optical density (OD) was measured on an automated spectrophotometric EL 340 multiplate reader (Bio-Tek Instruments Inc., USA) using test and reference wavelengths of 570 and 630 nm, respectively. The cytotoxic concentration/dose that killed 50% of the cells (IC₅₀) was determined from the absorbance (OD) versus concentration curve.

Determination of Morphological Changes

Untreated cells, MDA-MB-123 cells treated with 120, 100, 60 and 40 µg/mL of BA, and HL-60 cells treated with 140 µg/mL of BA for 24, 48 and 72 h were viewed under an inverted light microscope. Apoptotic characteristics were identified by the appearance of cell shrinkage, nuclear condensation, and/or the presence of membrane-bound apoptotic bodies. Necrotic characteristics were identified by the appearance of irregular clumping of chromatin, swelling of all cytoplasmic compartments and focal disruption of membranes.

Determination of the Mode of Cell Death

DNA fragmentation analysis

DNA from the untreated and BA-treated MDA-MB-123 cells (120, 100, 60 and 40 µg/mL) for 24, 48 and 72 h were isolated using the Apoptotic DNA Ladder Detection Kit (Chemicon, USA) and the manufacturer’s established methods. Agarose gel electrophoresis was carried out to analyse DNA fragmentation. A HindIII digest of lambda DNA was used
as a marker for size comparison. The agarose gel was then stained with ethidium bromide and the DNA visualised via UV illumination.

RESULTS AND DISCUSSION

Cytotoxicity of BA

In this study, BA was found to be cytotoxic to HL-60 and MDA-MB-231. The cytotoxic effects of BA against the MDA-MB-231 and HL-60 cells were examined using an MTT assay. The degree of cytotoxicity is defined as the concentration that reduces the cell number by 50% as compared to the untreated cells (IC<sub>50</sub>). The IC<sub>50</sub> values summarised in Table 1 shows that the IC<sub>50</sub> value of BA against MDA-MB-231 cells was lower than that of the HL-60 cells, indicating that the MDA-MB-231 cells were more sensitive to BA than the HL-60 cells. In particular, BA was markedly toxic to HL-60 cells at higher concentrations (134 μg/mL).

Table 1: Cytotoxicity of betulinic acid against MDA-MB-231 and HL-60 cell lines based on the IC<sub>50</sub> value determined by the MTT assay.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)a</th>
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<tr>
<td>Human mammary carcinoma (MDA-MB-231)</td>
<td>58 ± 3.06</td>
</tr>
<tr>
<td>Human promyelocytic leukaemia (HL-60)</td>
<td>134 ± 4.62</td>
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a The IC<sub>50</sub> value was expressed as the mean ± SD, determined from the results of MTT assay in triplicate experiments.

It has been stated that besides the type of drug, its concentration and its time-course, the mechanism of cell death depends on the type of cell line used for the study (Lennon, Martin and Cotter 1994). Previously, the compound has also been shown to be cytotoxic to human leukaemia cells (HL-60, U937 and K562), melanomas (G361, SK-MEL-28) and neuroblastomas (GOTO and NB-1) with the IC<sub>50</sub> values ranging from 1.1 to more than 20 μM (Hata et al. 2003). The reported IC<sub>50</sub> value towards HL-60 was far lower (6.6 μM) from what we have achieved in our study (293 μM), possibly due difference in the purity of BA and batch-to-batch variation of the cell line used.
Fig. 2: Different features of MDA-MB-231 cells after treatment with betulinic acid at 120 µg/mL (200 x magnification). A: The normal viable cell with a fibroblast-like shape was attached to the surface of the flask. B: The signs of the earliest stage of apoptosis. The cell was detached from the surface of the plate and showed cytoplasmic condensation and compaction of the nucleus. C: Blebbing of the cell. D: A membrane-bound, apoptotic body that was separated from the mother cell. E: A cell undergoing secondary necrosis. F: A necrotic cell showing focal disruption of the cell membrane.

Effects of BA on Cell Morphology in MDA-MD-231 and HL-60 Cells

MDA-MB-231 cells showed different features of apoptosis and necrosis when treated with BA (Fig. 2). The dead cells rounded up, detached from the surface of the flask and shrunk (Fig. 2). The features of MDA-MB-231 cells treated with various concentrations of BA (40, 60, 100 and 120 µg/mL) and the untreated cells (control) at 24 hours are shown in Figure 3. The number of attached cells decreased with an increase in the concentration of BA. Normal viable cells were fibroblast-like and were attached to the surface of the flask (Fig. 3E). The dead cells were detached from the surface and appeared oval or round in shape. The number of non-viable cells was prominent at the highest concentration (120 µg/mL) (Fig. 3A). Figure 4 shows the BA-treated MDA-MB-231 cells (40, 60, 100 and 120 µg/mL) and the untreated cells (control) at 72 h. The number of cells of all the treated groups was obviously less than the control. The number of non-viable cells was prominent at the highest concentration (120 µg/mL). In general, the number of attached cells (normal viable healthy cells) decreased as the concentration of the compound increased.
In the case of HL-60, the unaffected cells were spherical with smooth cell surface (Fig. 5B, 5D and 5F). The cells treated with BA at concentration of 140 μg/mL for 24, 48 and 72 h showed nuclear condensation and formation of apoptotic bodies (Fig. 5A, 5C and 5E). The number of cells in the treated groups was relatively low compared to the controls. They blebbed and formed apoptotic bodies. During apoptosis, cells initially rounded up and detached from the flask and their neighbours. This is followed by condensation of cytoplasm, dissolution of the nuclear envelope and separation of the nucleus into distinct fragments that ultimately bring about the collapse of the cell into several small intact vesicles (apoptotic bodies) (Kerr, Wyllie and Currie 1972). The remarkable phenotypic alterations of apoptotic cells are caused by the destruction of the normal nuclear architecture and the cleavage of chromatin during the cell demolition process. The collapse of the nucleus is thought to be due to destabilisation of the nuclear envelope as a consequence of lamin proteolysis. This results in loss of the matrix attachment regions-points at which the chromatin is attached to the nuclear envelope, causing the chromatin to compact (Martin and Diego 1997). Some cells with characteristics of necrosis were observed after treatment with the highest concentration of BA, but the incidence appeared to be very low.

Effects of BA on DNA Fragmentation in MDA-MD-231 Cells

To confirm the induction of apoptosis by BA, DNA fragmentation analysis was used as the parameter for assessment. Ladder-like pattern was visible on agarose gel from all concentrations of BA-treated MDA-MB-231 cells after 72 h (Fig. 6). Only DNA from cells treated with 40 μg/mL of the compound remained intact as that in the controls, after exposure to a shorter period of time (24 hours). After treatment with higher concentrations of BA (120, 100 and 60 μg/mL), DNA smearing (slight DNA degradation) was observed (data not shown). After 72 h, treatments with all concentrations of BA (40, 60, 100 and 120 μg/mL) caused DNA fragmentation in MDA-MB-231 cells, producing fragments of multiples of 200 base pairs (bp) that appeared as distinctive ladder-like pattern on agarose gel, one of the prominent biochemical hallmarks of apoptosis (Fig. 6).
Fig. 3: MDA-MB-231 cells incubated for 24 h in the presence of varying concentrations of betulinic acid: A: 120 µg/mL, B: 100 µg/mL, C: 60 g/mL, D: 40 µg/mL, and in the absence of betulinic acid E: The cells were detached from the surface of the flask after exposure to betulinic acid (200X magnification).
Fig. 4: MDA-MB-231 cells incubated for 72 h in the presence of varying concentrations of betulinic acid: A: 120 μg/mL, B: 100 μg/mL, C: 60 μg/mL, D: 40 μg/mL, and in the absence of betulinic acid E: Dead cells were detached from the surface of the flask after exposure to betulinic acid (100X magnification).
Fig. 5: HL-60 cells incubated for 24 h (A, B), 48 h (C, D) and 72 h (E, F) in the presence of 140 μg/mL of betulinic acid (A, C, E) and the absence of betulinic acid (B, D, F). HL-60 cells showed membrane blebbing and apoptotic bodies (arrow) after exposure to betulinic acid (200X magnification).
The ladder-like pattern is one of the hallmarks of apoptosis (Kerr, Wyllie and Currie 1972). The DNA fragmentation is caused by the activation of an endogenous nuclear endonuclease, which selectively and distinctively cleaves the double-stranded nuclear DNA at sites located between nucleosomal units (linker DNA), generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal discrete multiples of an approximately 180 bp subunit. The degraded DNA produces a so-called “ladder-like” pattern when run on DNA agarose gel electrophoresis (Wyllie 1981; Duke, Chernevak and Cohen 1983; Wyllie et al. 1984; Arends and Wyllie 1991). It has been suggested that DNA digestion in apoptotic cells is an ordered progression, which may involve the collaborative action of a number of endonucleases and proteases, and that the nature of the endonucleases activation may be cell-type specific (Martin and Diego 1997).

The induction of apoptosis by BA is indeed not new. The compound is known to be a selective apoptosis-inducing reagent for numerous cancerous cells such as melanoma, neuroblastoma and brain tumour cell, but not for normal cell lines (Zuco et al. 2002). It has been reported that BA is a prototype cytotoxic agent that triggers apoptosis via a direct effect on mitochondrial permeability transition, instead of activation of ligand-receptor systems such as CD95 (Fulda et al. 1997). Mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome c or apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the cytosol, where they can activate caspases and endonucleases (Fulda et al. 1998). The characteristic of selectively inducing apoptosis suggests that BA might represent a potent substance for future cancer chemotherapy (Hata et al. 2003) due to the fact that apoptosis is unique type of cell death that differs from necrotic cell death. Apoptosis is always regarded as an ideal way to destroy damaged cells, and an agent that can induce apoptosis is preferable for the management and therapy of cancer.
Fig. 6: DNA fragmentation analysis of MDA-MB-231 cells treated with various concentrations of betulinic acid for 72 h.

Lane M: Marker (HindIII digest of lambda DNA)
Lane 1: control (MDA-MB-231 cells untreated with betulinic acid)
Lane 2: MDA-MB-231 cells treated with 40 μg/mL of betulinic acid
Lane 3: MDA-MB-231 cells treated with 60 μg/mL of betulinic acid
Lane 4: MDA-MB-231 cells treated with 100 μg/mL of betulinic acid
Lane 5: MDA-MB-231 cells treated with 120 μg/mL of betulinic acid

CONCLUSION

Based on this study, we conclude that BA was more cytotoxic towards MDA-MB-231 than HL-60, and that it induced apoptosis in MDA-MB-231.
ACKNOWLEDGEMENTS

This project was funded by Universiti Putra Malaysia.

REFERENCES


Betulinic Acid Was More Cytotoxic Towards


