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Induction of intrinsic apoptosis in leukaemia stem cells and *in vivo* zebrafish model by betulonic acid isolated from *Walsura pinnata* Hassk (Meliaceae)

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ABSTRACT

Background: Leukaemia stem cells (LSC) have been associated with disease relapse and chemotherapy resistance. Betulonic acid (BA), a pentacyclic lupane-type triterpenoid, was reported to exhibit cytotoxicity toward various cancer cells and to be capable of inducing intrinsic apoptosis in solid tumours. However, the *in vitro* and *in vivo* apoptotic effects of BA against LSC remain unknown.
Hypothesis/Purpose: We aimed to determine whether BA isolated from bark of *Walsura pinnata* Hassk (Meliaceae) has pro-apoptotic effects on LSC in *in vitro* and *in vivo* models.
Study design/Methods: The population of high purity LSC was isolated from the Kasumi-1 cell line using magnetic sorting and characterised by flow cytometry. Cell viability was assessed using the MTS assay to examine dose- and time-dependent effects. The colony formation assay was performed in MethoCult® H4435 enriched media. Apoptosis was analysed using Annexin-V and propidium iodide staining, mitochondrial transmembrane potential was studied using JC-1 staining, and expression of apoptosis related genes (*BAX*, *Bcl-2* and *survivin*) was evaluated by real time-polymerase chain reaction (RT-PCR). Caspase 3/7 and 9 activities were monitored through Promega Caspase-Glo® over a period of 24 h. The *in vivo* antileukaemia activity was evaluated using LSC xenotransplanted zebrafish, observed for DNA fragmentation from apoptosis by TUNEL assay.
Results: BA maintained its potency against the LSC population in comparison to parental Kasumi-1 cells (fold differences ≤ 1.94) over various treatment time points and significantly inhibited the formation of colonies by LSC. Apoptosis was triggered by BA through the upregulation of *BAX* and suppression of *Bcl-2* and *survivin* genes with the loss of mitochondrial transmembrane potential, leading to the activation of caspase 9 followed by downstream caspase 3/7. BA was able to suppressed leukaemia formation and induced apoptosis in LSC xenotransplanted zebrafish.
Conclusions: The results demonstrate that BA inhibited the proliferative and colonogenic properties of LSC. BA induced apoptosis in LSC through the mitochondria pathway and was effective in the *in vivo* zebrafish model. Therefore, BA could be a lead compound for further development into a chemotherapy agent against LSC.

Abbreviations: ALDH, aldehyde dehydrogenase; AML, acute myelogenous leukaemia; BA, betulonic acid; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; FBS, foetal bovine serum; HBSS, Hanks Balanced Salt Solution; IC_{50}, 50% inhibitory concentration; IMDM, Iscove's Modified Dulbecco's Medium; JC-1, 1st J-aggregate-forming cationic dye; LSC, leukaemia stem cells; MACS, magnetic-activated cell sorting; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); NMR, nuclear magnetic resonance; PI, propidium iodide; RPMI-1640, Roswell Park Memorial Institute-1640; RT-PCR, reverse transcription polymerase chain reaction; RNA, ribonucleic acid; RQ, relative quantity; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; TLC, thin layer chromatography.

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Introduction

Leukaemia refers to a group of malignant blood diseases arising from uncontrolled proliferation of immature leukocytes. Leukaemia can be classified into various types, such as lymphocytic or myelogenous leukaemia, depending on the affected blood type. Leukaemia can also be subdivided into acute leukaemia, characterized by a rapid increase in immature blood cells or chronic leukaemia, a result of excessive accumulation of relatively mature abnormal white blood cells. Acute myelogenous leukaemia (AML) is a type of malignant blood disease of immature leukocytes derived from the myeloid (Krause and Etten, 2007). Unlike solid tumours, treatment for leukaemia relies heavily on chemotherapy. Current standard chemotherapy for AML combines cytarabine and an anthracycline that was developed 40 years ago. Although this regimen is able to achieve complete remission in almost 70% of patients, overall patient survival within 5 years is approximately 45% (Roboz, 2011). Recent years have witnessed advances in our understanding that leukaemia stem cells (LSC) are the cause of resistance toward traditional chemotherapy, resulting in cancer relapse and mortality (Rico et al., 2013). A rare population of stem cells with expression of cell surface markers $CD34^+$ and $CD38^-$ was reported initially by Lapidot et al. (1994) as the driver of leukaemia in AML patients. Later, others supported their findings that leukaemia cells with the surface markers $CD34^+CD38^-$ are enriched in LSC (Gal et al., 2006) and patients with more than a 3.5% LSC population in their blood samples have a shorter average relapse free period (5.6 months) compared to patients with a lower LSC population (16 months) (van Rhenen et al., 2005). Apart from surface markers, internal markers, such as aldehyde dehydrogenase (ALDH) activity, is also associated with poor prognosis. Gerber et al. (2012) noted that 6 out of 7 patients who experienced relapse had a $CD34^+CD38^-ALDH^+$ subpopulation in their blood samples. More importantly, the authors found patients with $CD34^+CD38^-ALDH^+$ cells were resistant to the current chemotherapy regime. LSC have the distinguishing characteristics of stem cells, such as self-renewal, quiescence, higher *in vivo* engraftment and colony forming potential, which set them apart from their progenitors (Jordan, 2002). Zebrafish are increasingly used as an *in vivo* model for investigating the efficacy of potential antitumor compounds, particularly against leukaemia (Corkery et al., 2011; Pruvot et al., 2011). The zebrafish's transparent body, undeveloped immune system within 5 days post-fertilization and the ability for xenotransplantation of leukaemia into the yolk sac without compromising its embryogenesis allows phenotypic evaluation of a compound's potency *in vivo* (Tat et al., 2013).

Natural products are an attractive source for drug discovery because they offer chemical diversity and novel compounds that are structurally distinct. In oncology, approximately 60% of current anticancer drugs originated from natural products or their derivatives (Gordaliza, 2007). Plants have survival mechanisms that secrete toxic chemicals to drive predators or other plants away, which can be a source of new anticancer drug discovery. Betulonic acid (3-oxo-lup-20(29)-28-oic acid, BA), which is found distributed in numerous parts of various plant species (Shang et al., 2014; Hsu et al., 2015; Patil et al., 2015; Sun et al., 2015), exhibits cytotoxic activity against breast, ovarian, and lung cancer cell lines (Baratto et al., 2013), cervical and thyroid tumours (Kommera et al., 2010) and selected human T cell lymphoblasts and leukaemias (Mukherjee et al., 2004). BA is a pentacyclic lupane-type triterpenoid that possess a keto and carboxyl group at C-3 and C-28, respectively, with a terminal double bond moiety at the C-29 position. BA differs from betulinic acid only at the C-3 position, where BA has a ketone instead of a hydroxyl with ß configuration. BA was reported to induce apoptosis through a mitochondrial signalling pathway (Yang et al., 2013). Apoptosis is a programmed natural cell death to remove dead and dysregulated cells. The process can be triggered by either the extrinsic pathway that involves the death receptors on the cell membrane or the intrinsic pathway through the mitochondria. The family of Bcl-2 proteins tightly regulates mitochondria mediated apoptosis, leading to the reduction of the mitochondria membrane potential. This reduction further activates caspase 9, triggering downstream caspase 3 or 7 and leading to DNA fragmentation at the end of the apoptosis process (Reed, 2003).

In this study, we isolated BA from the bark of *Walsura pinnata* Hassk (Meliaceae). *W. pinnata* is locally known as "*Lantupak mata kucing*" in Malaysia. This tree is an evergreen tree distributed widely in the tropical areas of Southeast Asia, such as Malaysia, Thailand and Indonesia. This species is found in lowlands and hills up to 600 m above sea level (Ridley, 1952). To the best of our knowledge, there is no report on the activity of BA against LSC. Because LSC are associated with disease relapse and reduced survival rate, we investigated the *in vitro* and *in vivo* apoptotic effects of BA on LSC.

Materials and methods

Reagents and chemicals

Dichloromethane, hexane, ethyl acetate, deuterated chloroform (deuteration degree min. 99.8%) and tetramethylsilane for extraction, isolation and characterization of compound was purchased from Merck (Branchburg, NJ, USA). All solvents used for the extraction and isolation processes were of analytical grade and distilled prior to use. Silica gel (Merck 60, 230–400 mesh) and TLC (Merck 60 GF_{254}) were obtained from Merck (Branchburg, NJ, USA). Positive controls (cytarabine and idarubicin) were acquired from Wako Pure Chemicals Industries Ltd (Osaka, Japan). The human acute myeloblastic leukaemia cell line (Kasumi-1) was obtained from Riken (Ibaraki, Japan). Cell culture media Roswell Park Memorial Institute-1640 (RPMI-1640), Iscove's Modified Dulbecco's Medium (IMDM), Hanks Balanced Salt Solution (HBSS), phosphate buffer saline (PBS), foetal bovine serum (FBS), L-glutamine, zebrafish E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$ and 0.16 mM $MgSO_4$) and 2-phenoxyethanol were obtained from Wako Pure Chemicals Industries Ltd (Osaka, Japan). Colony formation media, MethoCult® H4435 enriched, was purchased from Stemcell Technologies (Vancouver, Canada). BA or positive controls were dissolved in DMSO (Wako Pure Chemicals Industries Ltd, Osaka, Japan) as a stock solution of 400 mM and diluted with culture media to desired concentrations. Antibodies tagged with fluorescence probe (anti-CD34-PE and anti-CD38-PE/Cy7) were purchased from BioLegend (San Diego, CA, USA) whereas annexin-V and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, CA, USA). BD™ MitoScreen assay kit was obtained from BD Science (San Diego, CA, USA). MTS reagent (CellTiter 96 AQ_{ueous}® One Solution), Caspase-Glo® 3/7 (Z-DEVD-aminoluciferin) and Caspase-Glo® 9 (Z-LEHD-aminoluciferin) were purchased from Promega (Madison,WI, USA).

Plant materials

The plant materials of *W. pinnata* were collected over 243 km from Gua Musang, Kelantan to Kuala Lipis, Pahang, Malaysia. The sample was identified with the voucher specimen (KL 4571) and deposited at the Herbarium of the Chemistry Department, Faculty of Science, University of Malaya.