UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECTS OF BETULINIC ACID AND ITS DERIVATIVES ON HUMAN MYELOID LEUKEMIA (HL-60) CELL LINE

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By

NUR HANA BINTI FAUJAN

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in Institute of Bioscience to the School of Graduate Studies of Universiti Putra Malaysia

December 2008

UPM

DEDICATION

This thesis is dedicated to my beloved husband Khairul Syahmi bin Kamso and my lovely baby boy Muhammad Ahnaf. My deepest gratitude and love to my dear parents Associate Professor Dr Faujan b. H. Ahmad @ Amat and Pn. Samilah Kutim. To my all siblings and in-laws, thank you enough for your love and support.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of requirement for the degree of Master of Science

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December 2008

Chairman : Prof Dr Abdul Manaf Ali, Ph. D

Institute : Institute of Bioscience

Betulinic acid (BA) is a triterpene from bark extracts of *Melaleuca cajuputi* Powell (Myretaceae) a Malaysian plant. The cytotoxic effects of betulinic acid (BA) and its four synthetic derivatives that has been modified at C-3 hydroxy group of BA (betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethylsuccinyl)-betulinic acid (BAES), 3-*O*-succinyl-betulinic acid (BASUC) and betulinic acid benzoate (BCL) were screened on human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B), human cervical epithelial carcinoma (HeLa) human breast adenocarcinoma (MCF-7), mouse skin melanoma (B16), human glioblastoma (DBTRG0.5MG) cancer cell lines. Several derivatives elicited cytotoxicity as assessed by 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Betulinic acid acetate (BAAC) was the most effective among the betulinic acid

derivatives. It had the most potent cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC), suggesting its action is specific for tumor cells. This study was focused on HL-60 that showed the most sensitive cell line after 72 hours of treatment with all betulinic acid derivatives. BA and BAAC inhibit HL-60 cell line after 72 hours treatment with IC_{50} values of 2.60 and 1.38 µg/mL, respectively. On microscopic examination, both compounds exhibited characteristic morphological features of apoptosis, such as cell shrinkage and formation of apoptotic bodies. Fluorescent staining with acridine orange (AO) and propidium iodide (PI) revealed distinct chromatin condensation and nuclear fragmentation. The internucleosomal DNA fragmentation was confirmed by the pattern of DNA laddering into fragments with multiples of 180-220 base pairs detected in agarose gel electrophoresis. The induction of apoptosis was also confirmed by flow cytometric analysis of the cell cycle. BA and BAAC were shown to induce a time dependent increase in the sub G_1 peak indicating apoptotic phenomenon as obtained from the DNA content histogram analysis. BA and BAAC were marked as cytotoxic agent induced by apoptosis.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN SITOTOKSIK OLEH BETULINIK ASID DAN SEBATIAN TERBITANNYA TERHADAP JUJUKAN SEL LEUKEMIA MEILOSITIK MANUSIA (HL-60)

Oleh

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Betulinik acid (BA) merupakan sebatian triterpen yang diekstrak daripada *Melaleuca cajuputi* Powell sejenis tumbuhan di Malaysia. Kesan sitotoksik bagi betulinik asid (BA) dan empat sebatian terbitan sintetiknya yang telah diubahsuai pada C-3 kumpulan hidroksi bagi BA (betulinik asid acetat (BAAC), 3-*O*-(2',2'-dimetilsuccinil)-betulinik asid (BAES), 3-*O*-succinil-betulinik asid (BASUC) dan betulinik asid benzoat (BCL) telah diuji terhadap jujukan sel-sel kanser iaitu sel leukemia meilositik manusia (HL-60), leukemia T-limfoblastik manusia (CEM-SS), leukemia meilomonositik tikus (WEHI-3B), kanser servik epitelial manusia (HeLa), kanser payudara manusia (MCF-7), kanser kulit tikus (B16) dan kanser otak glioblastoma manusia (DBTRG0.5MG). Asai 3-[4,5-dimetiltizol-2-il]-2,5-difeniltetrazolium bromida (MTT) menunjukkan beberapa sebatian

terbitan mempamerkan kesan ketoksikan. Betulinik asid asitat (BAAC) merupakan sebatian yang paling efektif di antara sebatian terbitan betulinik asid yang lain. Sebatian ini mempunyai kesan ketoksikan yang sangat berpotensi terhadap leukemia meilositik manusia (HL-60), leukemia T-limfoblastik manusia (CEM-SS), leukemia meilomonositik tikus (WEHI-3B), kanser servik epitelial manusia (HeLa) dan tiada kesan sitoktoksik pada sel darah periferi manusia (PBMC), menunjukkan bahawa sebatian ini hanya bertindak pada sel kanser sahaja. Kajian ini telah ditumpukan pada HL-60 yang merupakan jujukan sel yang paling sensitif selepas 72 jam rawatan dengan semua sebatian terbitan betulinik asid. BA dan BAAC merencatkan pertumbuhan jujukan sel HL-60 selepas 72 jam rawatan dengan nilai IC_{50} masing-masing pada 2.60 and 1.38 μg/mL. Kedua-dua sebatian ini menunjukkan ciri-ciri apoptosis seperti pengecutan sel dan pembentukkan badan apoptotik apabila dilihat melalui mikroskop. Pembahagian kondensasi kromatin dan pemecahan nuclear telah dilihat melalui pewarnaan fluoresen akridin oren (AO) dan propidium iodin (PI). Pemecahan DNA internukleosom telah disahkan oleh pembentukan tangga DNA kepada serpihan dengan susunan 180 – 220 bp dikesan pada gel agaros elektroforesis. Penentuan apoptosis ini juga telah dikenalpasti pada kitaran sel menggunakan analisis flow sitometrik. Peningkatan puncak sub G_1 pada BA dan BAAC bergantung pada masa menerangkan fenomena apoptotik seperti yang dihasilkan oleh kandungan DNA pada analisis histogram. BA dan BAAC telah ditandai sebagai agen sitotoksik yang dicetuskan oleh apoptosis.

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support and encouragement and the joy they have brought to my life. Finally, I thank God for having blessed me with a wealth of family and friends that is truly beyond measure.

I certify that a Thesis Examination Committee has met on **16 December 2008** to conduct the final examination of Nur Hana binti Faujan on her thesis entitled "Cytotoxic Effects of Betulinic Acid and its derivatives on Human Myeloid Leukemia (HL-60) Cell Line" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

NUR HANA BINTI FAUJAN

Date: 27 April 2009

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LIST OF ABBREVIATIONS

ADP	adenosine diposphate
ALL	acute lymphblastic leukemia
AML	acute myeloblastic leukemia
ANLL	acute nonlymphocytic leukemia
AO	acridine orange
APL	acute promyelocytic leukemia
ATCC	american type culture collection
ATRA	all-*trans* retinoic acid
BA	betulinic acid
BAAC	betulinic acid acetate
CD_{50}	half maximal cytotoxic dose
CEM-SS	human T4-lymphoblastoid
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CR	complete remission
DBTRG0.5MG	human brain glioblastoma
DIC	disseminated intravascular coagulation
DMEM	dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid

DOX	doxorubicin
EC_{50}	half maximal effective concentration
ECS	endothelial cell specificity
EDTA	ethylenedinitrilotetraacetic acid
FAB	French-American-British
HIV-1	human immunodeficiency virus type-1
HSV-1	herpes simplex virus type-2
IC_{50}	half maximal inhibitory concentration
MTT	methyl-thiazol-tetrazolium
NCI	national cancer institute
OD	optical density
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
Ph^1	Philadelphia chromosome
PI	propidium iodide
RCB	RIKEN cell bank
P-pg	P-glycoprotein
RNA	ribonucleic acid
ROS	reaction oxygen species
SD	standard deviation
TBE	tris-borate-EDTA
TI	therapeutic index
WBC	white blood cell

CHAPTER 1

INTRODUCTION

Pentacyclic triterpenes have a wide spectrum of biological activities and some of them may be useful in medicine (Akihisa *et al.,* 1996). Betulinic acid (3β-hydroxy-lup-20(29)-ene-28-oic acid), an example of a pentacyclic triterpene is widely distributed in plant kingdom (Maurya *et al.,* 1989). This compound can be chemically derived from betulin, a substance found in the outer bark of white birch tree *Betula alba* (Pisha *et al.,* 1995). Some biological activities have been ascribed to betulinic acid, with includes anti-inflammatory, anti-tumor (Mukherjee *et al.,* 1997), anti-viral (De Clercq, 1995), anti-neoplastic (Fulda *et al.,* 1999) and anti-plasmodial (Ziegler *et al,* 2004).

Betulinic acid exerts a selective anti-tumor activity on cultured human melanoma (MEL-2) cells (Pisha *et al.,* 1995) and malignant brain tumor (Fulda *et al.,* 1999). This compound also showed inhibitory effect on leukemia (HL-60, U937 and K562) and neuroblastoma (GOTO and NB-1) cell growth (Hata *et al.,* 2003). BA has been specifically kills neuroectodermal tumor cells (Fulda *et al.,* 1997). The studies on human neuroblastoma (SHEP) cell line have revealed that BA acts on mitochondria without affecting cell surface receptor and induced apoptosis in cells, but not in lymphoid cell lines (Fulda *et al.,* 1998). The anti-tumor activity of betulinic acid has been reported to other human neoplastic cell lines including lung carcinomas, ovarian and cervical carcinomas (Zuco *et al.,* 2002).

The anti-viral properties of betulinic acid were also confirmed in clinical trials (De Clercq, 2000). Betulinic acid also has antiviral properties against influenza A and herpes simplex type 1 (HSV-1, strain 1C) viruses *in vitro* (Baltina *et al.,* 2003). Betulinic acid has also been shown to suppress ECHO 6 virus reproduction (Pavlova *et al.,* 2003). The isolated betulinic acid from leaves of *Syzigium claviflorum (Mytaceae),* exhibited inhibitory activity against human immunodeficiency virus type 1 (HIV-1) replication in H9 lymphocyte cells (Fujioka *et al.,* 1994).

Betulinic acid has been known to induce apoptosis selectively in tumor cells lines, but not on normal cell lines (Zuco *et al.,* 2002). The favorable therapeutic index from the lack of toxicity towards normal cells suggested betulinic acid to be an attractive and promising anti-tumor agent (Pisha *et al.,* 1995). This feature makes betulinic acid unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine and vincristine. All these anti-tumor compounds are very toxic and inhibit replication of both cancer and normal cells (Zuco *et al*., 2002).

In this project, the potential of betulinic acid isolated from a Malaysian plant as an anti-cancer agent on human myeloid leukemia cell line will be investigated. Here, the *in vitro* cytotoxic activity of betulinic acid and its derivatives will be assessed using leukemic cell lines and compared with conventional anti-neoplastic drug (doxorubicin).

Therefore, the objectives of this study are:

1. To determine the cytotoxic effect of betulinic acid and betulinic acid derivatives on different cancer cell lines.
2. To determine the IC_{50} values of betulinic acid and betulinic acid acetate on human myeloid leukemia, HL-60.
3. To investigate the effect of betulinic acid and betulinic acid acetate on the proliferation, morphological changes and mode of cell death on human myeloid leukemia, HL-60.
4. To compare the cytotoxic effect of betulinic acid and betulinic acid acetate with the conventional anti-neoplastic drug (doxorubicin).

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a heterogeneous group of diseases, characterized by uncontrolled growth of cell population (Griffith *et al.,* 1996). Continuous division of these cells results in the formation and growth of tumors. Tumors are classified with reference to a number of criteria including their behavior, their appearances and their origins (Evans, 1991). Cancer cells escape from many of the normal homeostatic mechanism that control proliferation (Goodman, 1994).

Clinical experience indicates that there are two fundamental types of tumors, benign and malignant, which behave in different ways (Darnell *et al.,* 1986). Benign tumors remain localized and do not spread to different parts of body (Evan, 1991). Malignant tumors do not remain localized and encapsulated. They invade surrounding tissues, get into the body's circulating system and set up areas of proliferation away from the site of their original appearance (Darnell *et al.,* 1986).

Most tumors contain alterations in both tumor-suppressor genes and oncogenes, suggesting that the lost of a tumor-suppressor genes function within a cell must be accompanied by the conversion or activation of proto-oncogenes into an oncogene before the cell becomes fully malignant (Becker and Deamer, 1991). Proto-oncogenes and

tumor-suppressor genes encode many kinds of proteins that help control cell growth and proliferation. Mutations in these genes can contribute to the development of cancer (Lodish *et al.,* 2000).

Cancerous tumor is the disease of malignant tumors. Malignant tumors or cancer are aggregates of cells caused by a series of chromosomal changes. Cancer cells often differ from their normal neighbors by a host of specific phenotypic changes, such as rapid division rate, invasion of new cellular territories, high metabolic rate and altered shape (Griffith *et al.,* 1996). Transformation of normal cells to malignant cells may be triggered by several factors including chemical exposure, viruses and radiations. It also can occur deliberately under cellular control during the processes such as meiosis or hypermutation (Lodish *et al.,* 2000).

Cancers originate within a single cell. Hence, cancers can be classified by the type of cell in which it originates and by the location of the cell as carcinomas, adenocarcinomas, malignant melanoma, sarcomas, leukemias, lymphomas or teratocarcinomas. Carcinomas originate in epithelial cells. Adenocarcinomas originate from glandular epithelial tissue. Malignant melanoma arises in melanocytes, a pigmented epithelial cell. Sarcoma begins in the connective tissue of bone or muscle. Leukemias start in the bone marrow stem cells. Lymphoma is a cancer originating in lymphatic tissue. Teratocarcinomas begin within germ cells (Evan, 1991).

2.2 Leukemia

Leukemia is a malignant hematopoietic disease characterized by an uncontrolled proliferation and block in differentiation of hematopoietic cells (Rowley, 1998). These malignant cells can spread to the lymph nodes, spleen, liver and other tissues. Leukemia is broadly classified as acute or chronic referring to the type of cell affected and by the rate of cell growth, and of myeloid or lymphoid according to the type of cell that is multiplying abnormally. Acute leukemia signifying rapidly progressing disease with a presence of immature, blast cells, while chronic leukemia, denotes slowly progressing disease with more mature and well-differentiated cells. Some chronic leukemia may transform into an acute phase as "blast crisis" (Leonard, 1993).

The leukemias are classified in accordance with silent pathological features of the abnormal excessive hemopoietic cells (Tariq and John, 2002). They are four major subtypes of leukemia and several rare forms. The acute leukemias are divided into acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL). The chronic leukemias are divided into chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). Other, rare types of leukemia include hairy cell leukemia, sezary cell leukemia, plasma cell leukemia and the leukemia phase of lymphoma (Edward and Gregory, 2002).

2.2.1 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is characterized by the malignant transformation of myeloid stem cells in the bone marrow, which are in capable of normal differentiation and maturation (Tariq and John, 2002). This type of leukemia is also referred to as acute nonlymphocytic leukemia (ANLL) because nonlymphoid cells are affected (Lowenberg *et al.,* 1999). Since the normal hematopoiesis is organized hierarchically, the malignant transformation can occur at several levels. AML may arise in a stem cell capable of differentiating into cells of erythroid, granulocytic, monocytic and megakaryocytic lineages, or in a lineage-restricted stem cell (Tariq and John, 2002). AML is most common cancer among adults between the ages 63 to 65 years (Lowenberg *et al.,* 1999).

Most patients with AML present with signs and symptoms arising from a bone marrow failure and organ infiltration by leukemic cells. Occasionally, patients may present as a consequence of hyperleucostasis. The diagnosis of AML is made when more than 30 per cent of blast cells (myeloblasts) are found in bone marrow or peripheral blood (Tariq and John, 2002). This results in anemia, thrombocytopenia and granulocytopenia with obvious symptoms such as fatigue, fever, dizziness, infection and bleeding. This disease has a very rapid onset. Without treatment, a patient only rarely survives more than 6 to 12 months (Ghaddar and Estey 2003).

AML is classically subdivided into subtypes L0 through L7 based on the various degrees of differentiation and the lineage of cell maturation using the French-American-