UNIVERSITI PUTRA MALAYSIA

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

NUR HANA BINTI FAUJAN

IB 2008 8
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HUMAN MYELOID LEUKEMIA (HL-60) CELL LINE

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
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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NUR HANA BINTI FAUJAN

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

NUR HANA BINTI FAUJAN

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Betulinik acid (BA) merupakan sebatian triterpen yang diekstrak daripada Melaleuca cajuputi Powell sejenis tumbuhan di Malaysia. Kesaran 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 sitotoksik 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 merancak pertumbuhan jujukan sel HL-60 selepas 72 jam rawatan dengan nilai IC\textsubscript{50} masing-masing pada 2.60 and 1.38 \textmu g/mL. Kedua-dua sebatian ini menunjukkan ciri-ciri apoptosis seperti pengecutan sel dan pembentukan 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\textsubscript{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.
ACKNOWLEDGEMENTS

I am greatly indebted to my mentor and supervisor, Professor Dr. Abdul Manaf Ali. This work would not have been possible without the guidance, inspiration, and fatherly presence of my supervisor. I would like to express my gratitude to Dr. Muhajir Hamid and Dr. Noorjahan Banu Mohamad Alitheen for not only serving on my committee but also for providing fundamental insights and constructive criticisms of my work. Thanks for your helpful comments and suggestions. You have made this work better through your efforts.

I also wish to thank Mr Hasrol and Mrs Madiah for guiding me in the initial stages of my work. Mrs Intan Soraya, Ms Hazalina and Mrs Zainura have been constant sources of help in and around our laboratory. Thanks to all the lab members, Mrs Asmah, Mrs Najiah, Mrs Mashitoh, Mrs Ainul Fajariah, Ms Rohaya, Mrs Zuhaida Asra, Mrs Suhaila and Ms Azrina Begam. I am very grateful for your pleasant collaboration.

I owe my deepest gratitude to my husband, Khairul Syahmi for his constant inspiration, love and presence and to our son, Muhammad Ahnaf. I also would like to thank my parents Associate Professor Dr Faujan b. H. Ahmad @ Amat and Pn. Samilah Kutim and all my family especially my sisters Nur Huda, Nur Hani, Nur Hafizah, Nur Hidayah and Nur Haziqah and my brothers Nur Hadi, Nur Hakim and Nur Hariz for their
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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Date: 14 May 2009
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
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>APPROVAL SHEETS</td>
<td>viii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER

1 INTRODUCTION  

2 LITERATURE REVIEW

2.1 Cancer  

2.2 Leukemia

- 2.2.1 Acute Myeloid Leukemia (AML)  
- 2.2.2 Acute Lymphocytic Leukemia (ALL)  
- 2.2.3 Chronic Myeloid Leukemia (CML)  
- 2.2.4 Chronic Lymphocytic Leukemia (CLL)  

2.3 Acute Promyelocytic Leukemia (APL)

- 2.3.1 Human Promyelocytic Leukemia Cells (HL-60)  

2.4 Cell Death

- 2.4.1 Necrosis  
- 2.4.2 Apoptosis  

2.5 Apoptosis in Cancer Therapy  

2.6 Cytotoxicity

- 2.6.1 Cytotoxic Drug  
- 2.6.2 Cytotoxic Drug for Acute Promyelocytic Leukemia (APL) Treatment  

2.7 Betulinic acid

- 2.7.1 Betulinic acid derivatives  

3 MATERIALS AND METHODS

3.1 Cell lines

- 3.1.1 Maintenance of Cell Lines  

xii
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>French-American- British (FAB) classification of Acute Myeloid Leukemia</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>French-American- British (FAB) classification of Acute Lymphocytic Leukemia</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Rai’s Staging System for Chronic Lymphocytic Leukemia</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Binet Staging System for Chronic Lymphocytic Leukemia</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>The cytotoxicity data of betulinic acid (BA) and betulinic acid derivatives (BAAC, BAES, BASUC and BCL) against leukemia cell lines</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>The cytotoxicity data of betulinic acid (BA) and betulinic acid derivatives (BAAC, BAES, BASUC and BCL) against cancer cell lines</td>
<td>56</td>
</tr>
<tr>
<td>7</td>
<td>The cytotoxicity data of doxorubicin (DOX) betulinic acid (BA) and betulinic acid acetate (BAAC) against human myeloid leukemia (HL-60) and normal human lymphocytes (PBMCs)</td>
<td>58</td>
</tr>
<tr>
<td>8</td>
<td>The cell cycle distribution of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) in HL-60 cell lines</td>
<td>82</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sequence of ultra structural changes in apoptosis and necrosis</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Chemical structure of betulinic acid</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>A schematic conversion of Betulin to Betulinic acid</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>Chemical structure of betulinic acid and its derivatives</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>The percentage of HL-60 cell viability after treated with doxorubicin, betulinic acid and betulinic acid acetate were evaluated at 24, 48 and 72 hours by MTT assay</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>The percentage of PBMC cell viability after treated with doxorubicin, betulinic acid and betulinic acid acetate were evaluated at 24, 48 and 72 hours by MTT assay</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>The effect of doxorubicin, betulinic acid and betulinic acid acetate on HL-60 cells proliferation after treated with IC(_{50}) value at 0, 24, 48 and 72 hours by MTT assay</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>The effect of 0.20 and 30.00 (\mu)g/mL doxorubicin, 2.60 and 30.00 (\mu)g/mL betulinic acid and 3.50 and 30.00 (\mu)g/mL betulinic acid acetate on HL-60 cells proliferation at 0, 24, 48 and 72 hours treatment by MTT assay</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>Morphology features of HL-60 cells incubated with doxorubicin</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>Morphology features of HL-60 cells incubated with betulinic acid</td>
<td>67</td>
</tr>
<tr>
<td>11</td>
<td>Morphology features of HL-60 cells incubated with betulinic acid acetate</td>
<td>68</td>
</tr>
<tr>
<td>12</td>
<td>Morphology features of HL-60 cells incubated with doxorubicin after stained with acridine orange and propidium iodide</td>
<td>70</td>
</tr>
<tr>
<td>13</td>
<td>Morphology features of HL-60 cells incubated with betulinic acid after stained with acridine orange and propidium iodide</td>
<td>`71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Morphology features of HL-60 cells incubated with betulinic acid acetate after stained with acridine orange and propidium iodide</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>DNA ladder formation following exposure of HL-60 cells to doxorubicin, betulinic acid and betulinic acid acetate</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Flow cytometry detection of apoptosis by treatment of HL-60 cells with doxorubicin at 0.2 μg/mL</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Flow cytometry detection of apoptosis by treatment of HL-60 cells with betulinic acid at 2.6 μg/mL</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Flow cytometry detection of apoptosis by treatment of HL-60 cells with betulinic acid acetate at 3.5 μg/mL</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ADP  adenosine dipophosphate
ALL  acute lymphoblastic 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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>doxorubicin</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>ECS</td>
<td>endothelial cell specificity</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenedinitrilotetraacetic acid</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus type-1</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus type-2</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>MTT</td>
<td>methyl-thiazol-tetrazolium</td>
</tr>
<tr>
<td>NCI</td>
<td>national cancer institute</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Ph¹</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RCB</td>
<td>RIKEN cell bank</td>
</tr>
<tr>
<td>P-pg</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reaction oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
<tr>
<td>TI</td>
<td>therapeutic index</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell</td>
</tr>
</tbody>
</table>
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, ellipticine, 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).
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 incapable 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-