# Studies on the Anti-tumor Activity of Conjugated Linoleic Acid against Myeloid Leukemia

Lui Oi Lan

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# Abbreviations

| | |
|---|---|
| $\Delta\psi m$ | Mitochondria membrane potential |
| 10E, 12Z-CLA | *trans*-10, *cis*-12 CLA |
| $^3$H-TdR | [Methyl-$^3$H] thymidine |
| 8-Cl-cAMP | 8-chloro-cyclic-cAMP |
| 9E, 11E-CLA | *trans*-9, *trans*-11 CLA |
| 9Z, 11E-CLA | *cis*-9, *trans*-11 CLA |
| 9Z, 11Z-CLA | *cis*-9, *cis*-11 CLA |
| ABTS | 2, 2'-Azino-bis[3-ethyl-benzothiazoline-6-sulfonate |
| AFC | 7-Amino-4-trifluoromethyl coumarin |
| AGM | Aorta / gonad / mesonephros |
| AIF | Apoptosis-inducing factor |
| ALAT | Aspartate aminotransferase |
| ALL | Acute lymphocytic leukemia |
| AMC | 7-Amino-4-methyl coumarin |
| AML | Acute myeloid leukemia |
| ANT | Adenine nucleotide translocator |
| Apaf-1 | Apoptotic protease-activating factor |
| APL | Acute promyelocytic leukemia |
| APS | Ammonium persulfate |
| ara-C | Cytarabine |
| $As_2O_3$ | Arsenic trioxide |
| ASAT | Alanine aminotransferase |
| ATCC | American Type Culture Collection |
| ATRA | All-trans-retinoic acid |
| Bad | Bcl-2-antagonist of cell death |
| Bak | Bcl-2-antagonist/killer |
| Bax | Bcl-2-associated X protein |
| Bcl-$_{XL}$ | B cell lymphoma protein long isoform |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| CAD | Caspase-activated DNase |

| | |
|---|---|
| CARD | Caspase-activation recruitment domain |
| CD | Conjugated diene |
| Cdk | Cyclin-dependent kinase |
| cDNA | Complementary DNA |
| CKI | Cyclin-dependent kinase inhibitor |
| CLA | Conjugated linoleic acid |
| CLA-mix | A mixture of CLA isomers |
| CLL | Chronic lymphocytic leukemia |
| CLP | Common lymphoid progenitor |
| CM | Complete RPMI medium |
| CML | Chronic myelocytic leukemia |
| CMP | Common myeloid progenitor |
| CPF | Core binding factor |
| cpm | Counts per minute |
| CSF | Colony-stimulating factor |
| CSF | Cerebrospinal fluid |
| Cyt c | Cytochrome c |
| DEF-45 | DNA fragmentation factor-45 |
| DEPC | Diethyl pyrocarbonate |
| DHA | Docosahexaenoic acid |
| DHE | Dihydroethidium |
| DM | Diabetes mellitus |
| DMBA | 7,12-dimethylbenz(a)anthracene |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| dTTP | Deoxythymidine triphosphate |
| EDTA | Ethylenediamine tetra-acetic acid |
| EGCG | Epigallocatechin-3-gallate |
| EPA | Eicosapentaenoic acid |
| EPO | Erythropoietin |
| EPOR | Erythropoietin receptor |
| EtBr | Ethidium bromide |

| | |
|---|---|
| ETO | Eight-twenty-one |
| EtOH | Ethanol |
| FAB | French-American-British |
| FADD | Fas-associated protein with death domain |
| Fas-L | Fas ligand |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward scatter |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| G-CSF | Granulocyte colony-stimulating factor |
| GCSFR | Granulocyte colony-stimulating factor receptor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GMP | Granulocyte-monocyte progenitors |
| GRZB | Granzyme B |
| GSH | Glutathione |
| GVHD | Graft-versus-host disease |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| HDACi | Histone deacetylase inhibitor |
| HEPES | N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid |
| HI-FBS | Heat-inactivated fetal bovine serum |
| HMBA | Hexamethylene bisacetamide |
| HSC | Hematopoietic stem cell |
| i.p. | Intraperitoneally |
| IFN-γ | Interferon-γ |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IMM | Inner mitochondrial membrane |
| iNOS | Inducible nitric oxide synthase |
| LA | Linoleic acid |
| LDL | Low density lipoprotein |
| LIF | Leukemia inhibiting factor |

| | |
|---|---|
| LPL | Lipoprotein lipase |
| LSC | Leukemic stem cell |
| LT-HSC | Long-term hematopoietic stem cell |
| M-CSF | Macrophage colony-stimulating factor |
| M-CSFR | Macrophage colony-stimulating factor receptor |
| MDR | Multidrug resistance |
| MEP | Megakaryocyte-erythrocyte progenitor |
| MHC | Major histocompatibility complex |
| MkP | Megakaryocyte committed progenitor |
| M-MLV RT | Moloney murine leukemia virus reverse transcriptase |
| MPO | Myeloperoxidase |
| MPP | Multipotent progenitor |
| MPT | Membrane permeability transition |
| MRP | Multidrug resistance protein |
| MSE | Monocytic serine esterase |
| Mts1 | Metastasis specific 1 |
| MTT | Methylthiazoletetrazolium |
| NAC | N-acetylcysteine |
| NaOH | Sodium hydroxide |
| NaPB | Sodium phenylbutyrate |
| NE | Neutrophil elastase |
| NK | Natural killer |
| NOAEL | No Observed Adverse Effect Level |
| $O_2^-$ | Superoxide anion |
| OMM | Outer mitochondrial membrane |
| PARP | Poly (ADP-ribose) polymerase |
| PBS | Phosphate-buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PEC | Peritoneal exudate cell |
| PI | Propidium iodide |
| PM | Plain medium |
| PPAR | Peroxisome proliferator-activated receptor |
| PS | Phosphatidyl serine |

| | |
|---|---|
| PVDF | Polyvinylidene difluoride |
| Rb | Retinoblastoma protein |
| RNA | Ribonucleic acid |
| RNase A | Ribonuclease A |
| ROS | Reactive oxygen species |
| RT | Reverse transcription |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| S.E. | Standard error |
| SAHA | Suberoylanilide hydroxamic acid |
| SC | Solvent control |
| SCD | Stearoyl-CoA desaturase |
| SCID | Severe combined immunodeficient |
| SCT | Stem cell transplantation |
| SDS | Sodium dodecyl sulfate |
| SOD | Superoxide dismutase |
| SSC | Side scatter |
| ST-HSC | Short-term hematopoietic stem cell |
| TBE | Tris-Borate-EDTA |
| tBid | Truncated Bid |
| TCR | T cell receptor |
| TEMED | N,N,N',N'-Tetra-methylethylenediamine |
| TG | Thioglycollate |
| TGF-β | Transforming growth factor-β |
| TNF-α | Tumor necrosis factor-α |
| TPA | 12-0-tetradecanoylphorbol-13-acetate |
| TPO | Thrombopoietin |
| ZDF | Zucker diabetic fatty |
| γ-GT | γ-glutamyltransferase |

# ABSTRACT

Nowadays, people are more concerned with the nutritional and medicinal values of the dietary and natural products than at any other time in the human history. Since conventional methods for the treatment of malignant diseases, including chemotherapy and radiotherapy, have a number of limitations and are often accompanied with many adverse side effects on normal body cells, therefore, the need for developing novel approaches to cancer treatment is unequivocal.

Conjugated linoleic acid (CLA) refers to a group of positional and geometrical isomers of the omega-6 essential fatty acid, linoleic acid (LA) (C18:2). All *cis*- and *trans*- isomeric combinations of CLA have been virtually identified in food. CLA is natural product that is derived from ruminant animals. Among all the CLA isomers, the *cis*-9, *trans*-11 (9Z, 11E) CLA and the *trans*-10, *cis*-12 (10E, 12Z) CLA are the predominant isomers found in human diet. There are numerous physiological and pharmacological activities attributed to CLA, including anti-adipogenic, anti-atherosclerotic, anti-carcinogenic, anti-diabetogenic, anti-tumor, as well as immunomodulatory properties. CLA has been a target of active cancer research in recent years. Although results from the majority of studies are promising, the mechanisms by which CLA and its isomers exhibit their anti-tumor activities remain poorly understood, and in particular their effects on the proliferation, apoptosis, and differentiation of myeloid leukemia cells have not yet been thoroughly investigated.

In the present study, the anti-proliferative activity of a mixture of CLA isomers (CLA-mix) and various individual CLA isomers on different myeloid leukemia cell

lines were investigated. Our *in vitro* studies showed that CLA-mix and its isomers inhibited the growth of leukemia and lymphoma cell lines in a dose-dependent manner. Moreover, the *cis*-9, *cis*-11 CLA (9Z, 11Z-CLA) isomer was found to be the least potent isomer among other CLA isomers being investigated. It is also clear that the growth-inhibitory activity of CLA-mix on the murine myelomonocytic leukemia WEHI-3B JCS cells was not a direct result of the cytotoxic effect of CLA-mix. In addition, CLA-mix exhibited relatively low cytotoxicity in normal murine peritoneal macrophages. Moreover, treatment of WEHI-3B JCS cells *in vitro* with CLA-mix or the 9E, 11E-CLA isomer could significantly reduce the *in vivo* tumorigenicity of WEHI-3B JCS cells in the syngeneic BALB/c mice.

The anti-tumor effect of CLA on the leukemia WEHI-3B JCS cells is probably mediated through cell cycle arrest at the $G_0/G_1$ phase and induction of apoptosis. Mechanistic study demonstrated that both CLA-mix and the 9E, 11E-CLA up-regulated the expression of several cell cycle-regulatory genes, including the *p53* and the cyclin-dependent kinase inhibitor (CKI) $p21^{CIP1/WAF1}$ genes, whereas the expression of the *cyclin A* gene was down-regulated. On the other hand, CLA-mix, 10E, 12Z-CLA and 9E, 11E-CLA induced DNA fragmentation of the WEHI-3B JCS cells. Interestingly, the 9E, 11E-CLA was the most potent apoptotic inducer among all the isomers being investigated. Our results also showed that both CLA-mix and the 9E, 11E-CLA isomer could induce mitochondrial membrane depolarization in the WEHI-3B JCS cells. Using the RT-PCR technique`, it was found that the anti-apoptotic $Bcl\text{-}x_L$ gene in WEHI-3B JCS cells was down-regulated while the pro-apoptotic *Bak* and *Bad* genes were up-regulated upon treatment with CLA-mix or 9E, 11E-CLA. Furthermore, Western blotting experiments showed that both CLA-mix and the 9E, 11E-CLA isomer increased the protein expression of the death receptor

Fas and its ligand, Fas-L, in the CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells. These findings suggest that apoptosis triggered by CLA-mix and the 9E, 11E-CLA isomer signals through both the "intrinsic" and "extrinsic" apoptotic pathways. This was further confirmed by studying the activities of caspases-3, -8 and -9. Furthermore, CLA-mix and the 9E, 11E-CLA isomer also enhanced superoxide anion production in the WEHI-3B JCS cells. Interestingly, the generation of superoxide anions and the formation of DNA ladders were partially inhibited by antioxidants such as superoxide dismutase and N-acetylcysteine.

In addition to induction of cell cycle arrest and apoptosis, CLA-mix could trigger the terminal differentiation of the WEHI-3B JCS cells. CLA-mix was found to increase the cytoplasm/nucleus ratio and vacuolation of WEHI-3B JCS cells, which are characteristics of mature macrophages. In addition, CLA differentially enhanced the expression of macrophage differentiation antigens, such as Mac-1 and F4/80, but not the granulocyte differentiation antigen, Gr-1. CLA also induced monocytic serine esterase and endocytic activities which further support that CLA can induce monocytic rather than granulocytic differentiation of WEHI-3B JCS cells.

The molecular mechanisms by which CLA-mix can induce differentiation of myeloid leukemia cells remain elusive. Our findings showed that the expression of certain cytokine genes, such as TNF-$\alpha$, IL-1$\beta$ and IFN-$\gamma$, was up-regulated in WEHI-3B JCS cells upon exposure to CLA-mix. Nevertheless, the underlying mechanisms for CLA-mix-induced monocytic differentiation of myeloid leukemia cells still await further investigations.

# 撮要

現今，人們對日常食品和天然產品的營養和藥用價值都非常關注。由於傳統治療惡性腫瘤的方法如放射性治療和化療等都有其局限及副作用，因此發展出嶄新的治癌方法確有其明顯的需要。

共軛亞油酸爲ω-6 必需脂肪酸亞油酸的一組位置異構體及幾何異構體。實際上，所有共軛亞油酸的順式與反式等軸組合都已在食物中找到。共軛亞油酸是一種從反芻動物中提取的天然產品。在所有共軛亞油酸的異構體中，只有順式-9、反式-11 (9Z,11E)共軛亞油酸及反式-10、順式-12 (10E, 12Z)共軛亞油酸爲存在於人類食物的主要異構體。共軛亞油酸具有多種生理和藥理作用，當中包括抗脂肪形成、抗動脈粥樣硬化、抗致癌、抗糖尿、抗腫瘤以及免疫調控等特性。近年來，共軛亞油酸已成爲抗癌研究主要對象之一。時至今日，雖然很多研究都取得肯定的成果，不過在共軛亞油酸如何抑制腫瘤細胞生長的機制方面，科學家仍茫無頭緒; 尤其對骨髓性血癌細胞的增生、分化及細胞凋亡的作用上，仍未有深入的研究。

在本研究計劃中，我們會探討共軛亞油酸混合物以及個別的共軛亞油酸異構體如何對抗各種骨髓性血癌細胞株的增生。體外實驗結果顯示，共軛亞油酸混合物及其異構體均能在濃度依賴的情況下抑制了白血病和淋巴瘤細胞株的生長。此外，在眾多共軛亞油酸異構體的研究對象中，我們還發現了順式-9，順式-11 共軛亞油酸 (9Z, 11Z-共軛亞油酸)異構體其抑制血癌細胞增生的效力是最弱的。除此之外，實驗結果明確地展示了解共軛亞油酸混合物在鼠科骨髓性血癌 WEHI-3B JCS 細胞中，其抑制生長的活性並不是由共軛亞油酸的細胞毒直接引起的。另外，共軛亞油酸混合物對正常小鼠腹腔巨噬細胞並無顯著的細胞毒性作

用。除此之外，WEHI-3B JCS 血癌細胞在體外經過共軛亞油酸混合物及 9E, 11E-共軛亞油酸異構體的處理後，其在同基因 BALB/c 小鼠中的致癌能力明顯地下降。

共軛亞油酸在 WEHI-3B JCS 血癌細胞中的抗癌能力可能是透過誘發細胞週期停留在 $G_0/G_1$ 的時相及誘導細胞凋亡而引致的。機制研究顯示共軛亞油酸混合物及 9E, 11E-共軛亞油酸兩者均增加了數種細胞週期調整基因的表達，其中包括 *p53* 及 *p21* 基因，而減少了*細胞週期素 A* 基因的表達。在另一方面，共軛亞油酸混合物、10E, 12Z-共軛亞油酸及 9E, 11E-共軛亞油酸誘發了 WEHI-3B JCS 細胞中脫氧核糖核酸的斷裂。有趣的是 9E, 11E-共軛亞油酸是眾多異構體研究對象中最強力的細胞凋亡誘導者。實驗結果亦證明了共軛亞油酸混合物和 9E, 11E 共軛亞油酸異構體均可誘發 WEHI-3B JCS 細胞的線粒體膜出現去極化現象。透過使用反向轉錄酶聚合酶連鎖反應技術，我們發現在 WEHI-3B JCS 細胞中抗細胞凋亡的 *Bcl-$_{XL}$* 基因表達有下調現象，而促進細胞凋亡的 *Bak* 及 *Bad* 基因表達則在共軛亞油酸混合物或 9E, 11E 共軛亞油酸的處理下增加了。

此外，從 Western 蛋白質印迹實驗顯示，共軛亞油酸混合物和 9E, 11E 共軛亞油酸異構體均增加了 Fas 死亡受體及其配體 Fas-L 在 WEHI-3B JCS 細胞中的蛋白表達。這些結果反映了由共軛亞油酸混合物和 9E, 11E 共軛亞油酸異構體觸發的細胞凋亡是通過「固有」和「外在」兩條凋亡路徑傳遞訊號的。而半胱氨酸天冬氨酸酶(caspases)-3、-8 及 9 的活性被提高了，更進一步印證了這一點。此外，共軛亞油酸混合物及 9E, 11E 共軛亞油酸異構體更增加了在 WEHI-3B JCS 細胞中製造的超氧化物陰離子。令人感興趣的是，超氧化物陰離子的產生和脫氧核糖核酸梯帶的形成，均部分地受到超氧化物岐化酶(SOD)及 N-乙酰半胱氨酸

(N-acetylcysteine)等抗氧化物所抑制。

除誘發細胞週期停止及細胞凋亡外，共軛亞油酸混合物亦可觸發 WEHI-3B JCS 的細胞分化。我們發現 WEHI-3B JCS 細胞經共軛亞油酸混合物處理後其細胞質/細胞核比例及其空泡化均有所增加 - 這些均爲成熟巨噬細胞的特徵。另外，WEHI-3B JCS 細胞的巨噬細胞分化抗原(包括 Mac-1， F4/80 等)的表達，均有上調現象，而其顆粒細胞分化抗原如 Gr-1 的表達則沒有明顯改變。共軛亞油酸混合物亦誘發了 WEHI-3B JCS 細胞單核絲胺酸酯酶及內吞能力的增加，這進一步證實了共軛亞油酸混合物能誘發 WEHI-3B JCS 血癌細胞進行單核而非顆粒細胞分化這個說法。

到目前爲止，共軛亞油酸混合物所誘發的骨髓性白血病細胞分化的分子機制仍有很多地方尚待探討及發掘。我們的實驗結果顯示，共軛亞油酸混合物能增加 WEHI-3B JCS 細胞中某些細胞因子如 TNF-α、IL-1β及 IFN-γ的基因表達。儘管如此，在共軛亞油酸混合物所引致的骨髓性白血病之單核分化中，其未爲人知的機制，仍有待更深入的研究。

# Publications

**Full Papers:**

Lui OL, Mak NK and Leung KN. Conjugated linoleic acid induces monocytic differentiation of myeloid leukemia WEHI-3B JCS cells. (Submitted to the *International Journal of Oncology*)

Lui OL and Leung KN. Conjugated linoleic acid inhibits proliferation of myeloid leukemia WEHI-3B JCS cells through induction of apoptosis and modulation of cell cycle. (Manuscript in preparation)

Lui OL and Leung KN. T*rans*-9, *trans*-11 conjugated linoleic acid isomer induces apoptosis in myeloid leukemia cells through oxidative stress. (Manuscript in preparation)

**Abstract Papers:**

Leung KN, Lui OL and Mak NK. (2005) Effects of conjugated linoleic acid on the proliferation, differentiation and apoptosis of myeloid leukemia cells. An oral paper to be presented in the 18$^{th}$ Asia Pacific Cancer Conference which will be held in Seoul, Korea from September 7-9, 2005.

Lui OL and Leung KN. (2004) Immunomodulatory and differentiation-inducing activities of conjugated linoleic acid on myeloid leukemia cells. A poster paper presented in the 34$^{th}$ Annual Scientific Meeting of the Japanese Society for Immunology which was held in Sapporo, Japan from December 1-3, 2004.

Lui OL and Leung KN. (2004) Anti-proliferative, differentiation- and apoptosis inducing activities of conjugated linoleic acid (CLA) on myeloid leukemia cells. *International Journal of Molecular Medicine Supplement* **14**: S37.

Lui OL and Leung KN. (2004) Modulatory effects of conjugated linoleic acid on the proliferation, differentiation and apoptosis of myeloid leukemia cells. *Clinical and Investigative Medicine Journal Supplement* **27**: 61A.

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# CHAPTER 1

# GENERAL INTRODUCTION

# 1.1 Hematopoiesis and Leukemia

## 1.1.1 An Overview on Hematopoietic Development

Hematopoiesis occurs sequentially in distinct anatomical locations during development. Since most mature blood cells are short-lived, hematopoiesis assures continuous hematopoietic cell production throughout adult life (Rane and Reddy, 2002). In mammals, embryonic hematopoiesis begins in the yolk sac while definitive hematopoiesis occurs in the fetal liver and then switches to the bone marrow and spleen. In the mouse, both blood and endothelial progenitors first emerge in the extra-embryonic yolk sac blood islands at about embryonic day 7.5 (E7.5) (Haar and Ackerman, 1971). The yolk sac largely supports the production of primitive hematopoietic cells, consisting primarily of nucleated erythrocytes. Colonization of fetal liver by hematopoietic stem cells (HSC) derived from the yolk sac or the aorta/gonad/mesonephros (AGM) region of the embryo commences at around E10 or E11, and by E12 the fetal liver is the major site for hematopoietic cell development. Fetal liver HSC eventually migrate to the bone marrow at around E16 or E17 (Ikuta and Weissman, 1993), and the bone marrow becomes the predominant site for postnatal hematopoiesis which is continued into adult life (Zanjani *et al.*, 1993).

HSC contribute to the production of 200 to 250 billion ($2\text{-}2.5 \times 10^{11}$) erythrocytes, 150 to 200 billion platelets, and 100 to 150 billion neutrophils every day throughout human adulthood (Van Zant *et al.*, 1997). The complex hematopoietic system begins with an asynchronously dividing population of HSC (Cheshier *et al.*, 1999). These multipotent HSC are functionally defined by their unique capacity to differentiate into all mature blood cell types and maintain self-renewal activity. Self-renewal can be symmetrical, producing two daughter HSC, or asymmetrical,

resulting in one HSC and another downstream progeny, commonly known as a "progenitor" (Warner *et al.*, 2004). HSC can be classified as long-term HSC (LT-HSC), which are highly self-renewing cells that reconstitute an animal for its entire life span or short-term HSC (ST-HCS), which reconstitute the animal for a limited period (Passegue *et al.*, 2003). Mouse HSC which commit to differentiation pass through a phase of being ST-HSC that self-renew for six to eight weeks only, and then advance to the multipotent progenitor (MPP) stage. MPP self-renew for less than two weeks, but neither MPP nor ST-HSC are capable of dedifferentiating to LT-HSC (Shizuru *et al.*, 2005). LT-HSC give rise to various differentiated cells through a series of downstream progenitors, which have limited ability to self-renew and restricted capacity to differentiate along different cell lineages (Warner *et al.*, 2004). In general, the process of development from oligopotent progenitors to mature cells with specific functions involves the progressive loss of developmental potential to other lineages. The progenitor cells that commit to lymphoid lineage are known as common lymphoid progenitors (CLP). CLP give rise to progenitors of at least four cell types -- T lymphocytes, B lymphocytes, natural killer (NK) lymphocytes, and antigen-presenting dendritic cells (Manz *et al.*, 2001). On the other hand, the progenitor cells that commit to myeloid lineage are known as common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP), as well as megakaryocyte-erythrocyte progenitors (MEP) (Kondo *et al.*, 1997; Akashi *et al.*, 2000). Recently, mouse megakaryocyte committed progenitors (MkP) have been identified as a downstream progeny derived from MEP (Nakorn *et al.*, 2003).

Hematopoiesis is highly regulated by expression of genes in response to lineage-committed differentiation of HSC, depending on the hematopoietic micro-environment. Cytokines such as colony-stimulating factors (CSF) and

interleukins (IL) are crucial examples of the hematopoietic regulatory factors (Goldsby *et al.*, 2003). When mouse HSC are incubated with cytokines and/or ligands specific for their surface receptors, they enact gene expression profiles with appropriate lineage readout, i.e., myeloid and lymphoid cells through the stimulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), respectively. Apart from being the precursors of erythro-megakaryocytic and granulo-monocytic progenitors, the common myeloid progenitors also give rise to eosinophil progenitors and basophil progenitors, which subsequently differentiate into eosinophils in response to IL-5 and basophils in response to IL-4, respectively. For the erythro-megakaryocytic lineage, CMP give rise to erythrocytes and blood platelets in response to erythropoietin (EPO) and thrombopoietin (TPO) respectively; while for the granulo-monocytic lineage, CMP give rise to neutrophils in response to granulocyte colony-stimulating factor (G-CSF), or monocytes in response to macrophage colony-stimulating factor (M-CSF) (Zhu and Emerson, 2002). The monocytes, located in the circulation, would migrate to tissues where they are then called "macrophages". For the lymphoid lineage, the common lymphoid progenitors are generated through the stimulation of IL-3. Apart from being the precursors of NK lymphocytes and dendritic cells, as described just previously, CLP also give rise to T and B lymphocytes. Interleukins such as IL-7 and IL-2 have been documented to play important roles in the development and maturation of T and B lymphocytes (Howard and Hamilton, 2002). The conceptual hematopoietic trees are depicted in Figure 1.1.

Whether the differentiation of HSC through lineage-committed progenitors to mature effector cells occurs as the result of exogenous or intrinsic signals remains ambiguous, however, the molecular mechanisms in either case are revealed in the gene expression profiles of HSC and the downstream lymphoid and myeloid

progenitor cells (CLP, CMP, GMP, and MEP) along the differentiation hierarchy. Clusters of genes preferentially expressed in progenitors precede commitment to a particular lineage, and the shifts in the gene expression profiles correlate with the distinct potentials associated with each of the progenitors. At the CLP/CMP lineage checkpoint, HSC commit to the lymphoid lineage by shutting down expression of granulocyte/macrophage-affiliated genes [e.g. myeloperoxidase (MPO) and granulocyte colony-stimulating factor receptor (GCSFR)] and erythrocyte-affiliated genes [β-globin and erythropoietin receptor (EPOR)] (Akashi *et al.*, 2000), therefore preventing myeloid cell fates. It is interesting to note that myeloid differentiation is the default developmental pathway and that the gene expression programs required for lymphoid differentiation must be actively induced (Kondo *et al.*, 2003).

Under steady-state conditions, the majority of HSC is quiescent or divides slowly. Once they divide, they do so asymmetrically for the production of the entire complement of cells necessary to maintain blood production over time (Dick, 2003). Yet under certain circumstances, such as HSC transplantation, their divisions can be mostly symmetrical for a period of time to regenerate the stem cell pool before reverting to asymmetrical divisions (Warner *et al.*, 2004). Therefore, normal hematopoietic development greatly relies on the delicate balance between self-renewal and differentiation. When the processes of self-renewal and differentiation become deregulated or uncoupled, leukemias and other myeloproliferative and lymphoproliferative disorders can result. Leukemogenesis arises from clonal expansion of a single cell and is sustained by a leukemic stem cell (LSC). In fact, multiple acquired genetic changes must take place in order to convert a normal HSC into a LSC. LSC acquires mechanisms – altered self-renewal capacity, increased survival, arrested differentiation and telomere maintenance – through which

they can replicate indefinitely (Warner *et al.*, 2004). Frequently, transcriptions involve certain genes encoding transcription factors which have been reported to play a pivotal role in hematopoiesis. Therefore, aberrations in the transcriptional machinery appear to be one of the necessary mechanisms leading to leukemias. A representative target for mutations in human leukemia is the AML1 (Runx1) Runt domain-core binding factor β (CBFβ)-DNA ternary complex (Harada *et al.*, 2003). Core binding factor (CBF) is a transcription factor complex that consists of α- and β-units. CBFα binds DNA while CBFβ stabilizes the DNA-binding action of CBFα. By binding to DNA, CBF regulates the expression of a number of genes involved in hematopoiesis, which include GM-CSF, macrophage colony-stimulating factor receptor (M-CSFR), T cell receptor-α, -β, -δ, and -γ (TCR-α, -β, -δ, -γ), cell surface glycoprotein CD36, IL-3, granzyme B (GRZB), MPO, neutrophil elastase (NE) and metastasis specific 1 (mts1) genes (van der Reijden *et al.*, 1997). It is generally acknowledged that AML1 (acute myeloid leukemia-1), a Runt domain-containing transcription factor encoded by CBFα unit, is one of those genes most frequently involved in the chromosomal translocations of human leukemias as a result of point mutation in the transcription factor (Vradii *et al.*, 2005). The t(8;21) chromosomal translocation directs the fusion of two transcription factors, AML1 and ETO (eight-twenty-one or MTG8). The resulting chimeric fusion protein, AML1-ETO, is a dominant-negative inhibitor of CBF transcriptional regulation, which represses gene expression obligatory for normal hematopoiesis (Warner *et al.*, 2004). Therefore, blocked-differentiation of myeloid progenitors and maturation arrest at different stages during hematopoiesis are resulted, ultimately leading to human leukemias.

To conclude, hematopoiesis is a complex and highly regulated process through interactions of various signaling pathways. It is the process of blood cell formation,

which involves the proliferation of hematopoietic progenitors and their differentiation into mature erythrocytes, leukocytes, and platelets. There are catastrophic consequences to aberrant hematopoiesis including inborn error of metabolism, aplastic anemia, neutropenia, thrombocytopenia, or a combination of these cytopenias, myelodysplasia, myeloproliferative disorders, lymphoproliferative disorders, and hematologic malignancies including leukemia and lymphoma (Van Zant *et al.*, 1997; Smith, 2003). Among all these disorders, leukemia will be discussed in the next section in detail.

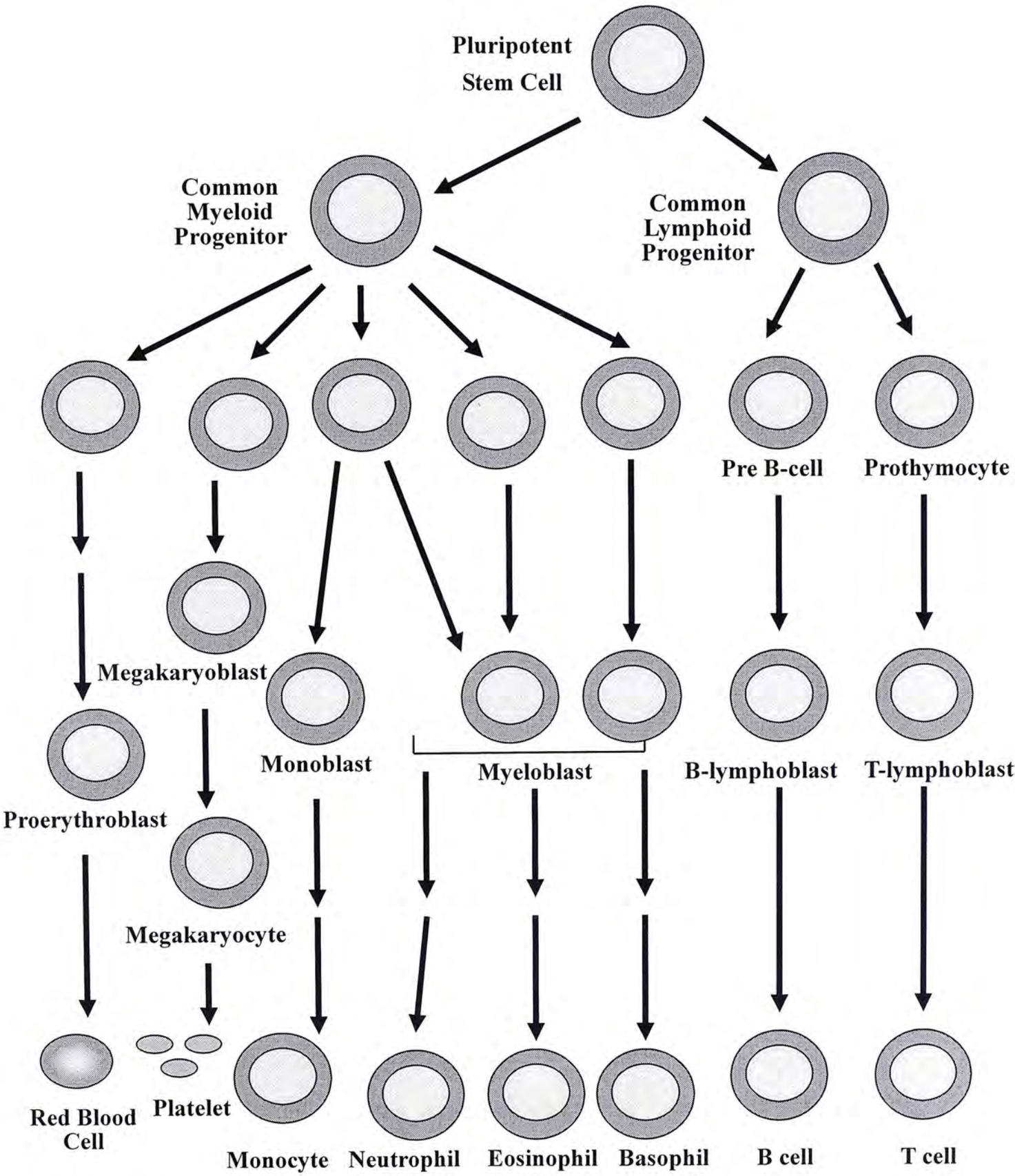



**Fig. 1.1: Hematopoiesis.** Different types of mature blood cells are developed from pluripotent hematopoietic stem cells. Generally speaking, throughout the whole developmental process, the proliferative capacity of the cells decreases with increasing degree of differentiation in their functions and number.
(Howard and Hamilton, 2002)

### 1.1.2 Leukemia

As discussed in section 1.1, the hematopoietic stem cells are capable of developing into two general types of blood cells - lymphocytes and myeloid cells. In the lymphoid lineage, the earliest lymphocyte is the lymphoblast, which can transform into lymphoblastic or lymphocytic leukemias (American Cancer Society, 2005). In this case, leukemias involve the lymphocytes of the white blood cells. In the myeloid lineage, the myeloid stem cell is the earliest antecedent that gives rise to white blood cells, red blood cells, or platelet-producing cells. Apart from leukocyte leukemias, leukemias also involve erythrocyte leukemias and megakaryocyte leukemias (American Cancer Society, 2005). Although erythrocyte leukemias and megakaryocyte leukemias are quite rare, we should never solely describe leukemias as being white blood cell diseases. In fact, leukemia is characterized by perturbation of the normal differentiation program with maturation arrest leading to an accumulation of immature cells (the blast cells) in the bone marrow (Warner *et al.*, 2004). Like other neoplasms, it arises from the neoplastic clonal expansion of a single cell.

Leukemia initiates in the bone marrow, yet it rapidly spreads into the circulation and eventually reaches lymph nodes, the spleen, the liver, the central nervous system, and other organs (American Cancer Society, 2005). General symptoms of leukemia include fatigue, weakness, weight loss, fever, loss of appetite, and less often, profuse bleeding from the gums and mucous membranes under the skin (Van Zant *et al.*, 1997; American Cancer Society, 2005), and most of which are resulted from a shortage of normal and functional blood cells comprising leukocytes, erythrocytes, and megakaryocytes. Although leukemia is typically thought to be a childhood disease, it strikes many more adults (Karen and Peterson, 2005). It is a complex disease with

many different types and subtypes, which will be discussed in the following section.

### 1.1.2.1 General Diagnostic Tests for Leukemia

Blood cell counts, blood examination, bone marrow tests, as well as various lab tests are the most common tests to find out if leukemia is present, what type of leukemia it is, and how well the disease is responding to specific treatments.

Blood cell counts can detect changes in the quantities of different blood cell types. Most people with leukemias will have excess yet non-functional white blood cells, insufficient red blood cells, and insufficient platelets (American Cancer Society, 2005). We can also classify the leukemia cells into specific types by looking at their size and shape under a microscope. The most immature cells are called "blasts", the number of blasts in the bone marrow is essential in telling if a person has leukemia.

There are two different bone marrow tests to diagnose for leukemias. The first one is bone marrow aspiration, through which a thin needle is used to draw up a small amount of liquid bone marrow (American Cancer Society, 2005). The second one is bone marrow biopsy, through which a small cylinder of bone and marrow is removed with a slightly larger needle (American Cancer Society, 2005).

Furthermore, there are precise lab tests which help to diagnose and classify leukemias, and these include cytochemistry, flow cytometry, immunocytochemistry, cytogenetics, and molecular genetic studies (American Cancer Society, 2005).

### 1.1.2.2 Classification and Epidemiology of Leukemia

Leukemias can be first classified by their onset rate and the degree of cellular maturation. As a result, there are acute leukemias and chronic leukemias. "Acute" refers to a condition where the leukemia cells grow rapidly, however, they are incapable of maturing properly (Hoffbrand *et al.*, 2001; American Cancer Society, 2005). In addition, many of these immature cells are blast cells, which are normally found in the bone marrow. If acute leukemias are not well treated, they could be fatal in a few months. On the contrary, "chronic" means that the leukemia cells look mature but they are not fully normal and functional. In chronic leukemias, the white blood cells generally do not fight infections as well as do normal white blood cells. The diseases have a gradual onset with a prolonged clinical course (Hoffbrand *et al.*, 2001; American Cancer Society, 2005). The second factor to consider in classifying leukemia is the type of bone marrow cells that are affected. If granulocytes or monocytes are involved, the leukemia is called myelocytic leukemia (also known as myeloid or myelogenous leukemia); if bone marrow lymphocytes are involved, the leukemia is called lymphocytic leukemia.

Therefore, by looking at whether a leukemia is acute or chronic, and whether it is myelocytic or lymphocytic, most cases of leukemias can be sorted into one of the four major types: acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), and chronic lymphocytic leukemia (CLL) (American Cancer Society, 2005). The key statistics and the risk factors for the four major types of leukemias are summarized in Table 1.1.

In 2005, there will be about 34,810 new cases of all types of leukemia in the United States, and acute leukemias contribute to about half of these cases. Among all,

AML is the most common leukemia. About 20% of adult AML cases are linked to smoking. In addition, chromosomal translocations, deletions, and inversions can also affect the development of AML. Translocations, which often occur in many cases of AML, can turn on oncogenes, shut down tumor-suppressor genes, or turn off genes that would normally help a cell to mature (American Cancer Society, 2005). For most types of cancers, they are given stages of I, II, III, or IV, based on the tumor size and how far from the original site the cancer has metastasized. Stages are useful in determining the prognosis (outlook for chances of survival) and guiding specific therapies (American Cancer Society, 2005). However, leukemia does not usually form a solid mass or tumor. Several years ago, an international congress was held to decide on the best system of classification of acute leukemias. This group of French, American, and British hematologists as well as pathologists decided that AML should be further divided into 8 subtypes, designated M0 through M7, based on the type of cells from which the leukemia developed. This system (the French-American-British or FAB system) was based on the microscopic appearance of the cells such as ultrastructural morphology, cytogenetics, immunophenotyping, and immunohistochemical markers (Hoffbrand *et al.*, 2001). Table 1.2 summarizes the FAB classification of AML.

ALL affects children and adults. Most leukemias in children are ALL, which occurs at age 3 to 6. Having at least 20% to 30% lymphoblasts, the earliest lymphocytes, in the bone marrow is generally required for diagnosis of ALL. The most significant factor that decides ALL outcomes is whether there are chromosomal translocations. The most common translocation seen in ALL is known as the "Philadelphia chromosome", which is an extra large chromosome 22 as a result of a translocation between chromosomes 9 and 22. It occurs in about 20% to 25% of ALL

cases and patients with such a translocation have a much worse outcome than those lacking it. Another translocation between chromosomes 4 and 11 also carries a poor outlook. This occurs in about 5% of the ALL cases (American Cancer Society, 2005). Other chromosome changes such as deletions and inversions can also affect ALL development, although they are much rarer. According to the FAB system, ALL should be divided into three subclasses as summarized in Table 1.2. The L3 subclass is also known as the Burkitt type leukemia.

CML is a type of leukemia that initiates in blood-forming cells of the bone marrow and invades the blood. It can also progress into a fast-growing acute leukemia that invades almost any organ in the body. The changes in DNA leading to cancer have been well illustrated in CML. "Philadelphia chromosome" is found in the leukemia cells of almost all patients with CML. The translocation contributes to the formation of an oncogene called bcr-abl, which acts to initiate leukemic cell growth and development (Randolph *et al.*, 2005). According to the American Cancer Society, CML can be categorized into three phases as summarized in Table 1.3.

There are two forms of CLL, one is very slow growing and the other is faster growing. In fact, the slow-growing CLL rarely needs to be treated, and the average survival for patients with this form of CLL is around 25 years. On the other hand, the latter form of CLL is a more serious disorder, and patients have an average survival of only about 6 to 8 years (American Cancer Society, 2005). CLL originates from a clone of B lymphocytes in 95% of patients while only 5% shows a T-lymphocyte origin. Each human cell contains 23 pairs of chromosomes, however there is often a deletion in chromosome from the leukemia cells of patients with CLL (Dickinson *et al.*, 2005). Although the chromosomal deletion often occurs in chromosome 11 or 13,

other chromosomes can also be affected. Moreover, an extra chromosome 12 can be sometimes found in CLL. There are two systems for staging CLL. The Rai classification is used more often in the United States, whereas the Binet system is used more widely in Europe. The Rai stages can be separated into low-, intermediate-, and high-risk categories. Stage 0 is considered low risk, stages I and II are considered intermediate risk, and stages III and IV are considered high risk (American Cancer Society, 2005). In the Binet system, CLL is classified according to the number of affected lymphoid tissue groups (neck lymph nodes, groin lymph nodes, underarm lymph nodes, spleen, and liver) and the presence of anemia or thrombocytopenia (American Cancer Society, 2005). Table 1.3 summarizes the Rai system and the Binet system for classification of CLL.

In Hong Kong, childhood cancer is the second leading cause of death among children. The incidence rate is about one per 10,000 children and around 120 to 150 new cases develop each year. Among different types of malignancy, leukemia is the most common childhood cancer in Hong Kong, with 36% of total malignant cases as reported in the most recent ten-year review (Children's Cancer Foundation, Hong Kong, 2005).

**Table 1.1: Key statistics and risk factors for the four major types of leukemias.**

| | AML | ALL | CML | CLL |
|---|---|---|---|---|
| Estimated new cases in 2005 in the US alone | 11,960 | 3,970 | 4,600 | 9,730 |
| Estimated no. of death in 2005 in the US alone | 9,000 | 1,490 | 850 | 4,600 |
| Average age of patients | 65 | 50 | 50 | 70 |
| Ratio of adult to children | Adult: 90%<br>Children: 10% | Adult: 67%<br>Children: 33% | Adult: 98%<br>Children: 2% | Adult only |
| * 5-year survival rate | Adult: 11%<br>Children: 40% | Adult: 56%<br>Children: 70% | 27% | 68% |
| Sex prevalence | Male | - | - | - |
| Risk factors | • Smoking<br>• Long-term exposure to high levels of benzene<br>• High-dose radiation<br>• Viral infection (e.g. HTLV-1) | • High-dose radiation<br>• Viral infection (e.g. HTLV-1) | • High-dose radiation | • Herbicides (e.g. Agent Orange)<br>• Insecticides |

* The 5-year survival rate refers to the percentage of patients who live at least 5 years after their cancer is found. In the United States, it varies according to the type of leukemia and the age of the patient.

(American Cancer Society, 2005; Karen and Peterson, 2005)

**Table 1.2: The French-American-British (FAB) system for classification of acute leukemias.**

| Type | Subtype | Sub-class |
|---|---|---|
| Acute Leukemia | Acute Myeloid Leukemia (AML) | • M0: undifferentiated myeloblastic, ~ 5%<br>• M1: myeloblastic with minimal maturation, ~ 15%<br>• M2: myeloblastic with granulocytic maturation, ~ 25%<br>• M3: promyelocytic with many granules, ~ 10%<br>• M4: myelomonocytic, ~ 25% (eosinophilia, ~ 5%)<br>• M5A: monoblastic; M5B: promonoblastic, ~ 10% totally<br>• M6: erythroblastic, ~ 5%<br>• M7: megakaryoblastic, ~ 5% |
| | Acute Lymphoblastic Leukemia (ALL) | • L1: T cell or pre-B cell; homogeneous small blasts with little cytoplasm; typically seen in children; ~ 30%<br>• L2: T cell or pre-B cell; heterogeneous larger blasts with variable amounts of cytoplasm; most often in adult; ~ 65%<br>• L3: B cell; homogeneous large blasts with vacuolated basophilic cytoplasm; rare; ~ 5% |

(Modified from Hoffbrand *et al.*, 2001; American Cancer Society, 2005)

**Table 1.3: Classification of chronic leukemias.**

| Type | Subtype | Phase / Stage |
|---|---|---|
| Chronic Leukemia | Chronic Myeloid Leukemia (CML) | • Chronic phase: fewer than 10% blasts (or 20% blasts and promyelocytes combined) in blood or bone marrow samples; patients usually have mild symptoms and respond to standard treatments<br>• Accelerated phase: more than 10% blasts (or 20% blasts and promyelocytes combined) but fewer than 30% blasts and promyelocytes in blood or bone marrow samples; the leukemia cells often have developed new chromosome changes in addition to the Philadelphia chromosome; symptoms and blood counts of patients are not as responsive to therapies as they are during the chronic phase<br>• Blast phase (also called acute phase or blast crisis): bone marrow and/or blood samples have more than 30% blasts and promyelocytes; the blast cells often spread to tissues beyond the bone marrow; CML has transformed into a very aggressive acute leukemia at this phase |
| | Chronic Lymphoblastic Leukemia (CLL) | <u>The Rai system:</u><br>• Stage 0: lymphocytosis is present (blood lymphocyte count is over 10,000 lymphocytes per $mm^3$ of blood); lymph nodes, spleen, and liver are not enlarged; red blood cell and platelet counts are near normal<br>• Stage I: lymphocytosis and enlarged lymph nodes; spleen and liver are not enlarged; red blood cell and platelet counts are near normal<br>• Stage II: lymphocytosis and enlarged liver or spleen; with or without enlarged lymph nodes; red blood cell and platelet counts are near normal<br>• Stage III: lymphocytosis and anemia; with or without enlarged lymph nodes, spleen, or liver; platelet counts are near normal<br>• Stage IV: lymphocytosis and thrombocytopenia; with or without anemia, enlarged lymph nodes, spleen, or liver<br><u>The Binet system:</u><br>• Stage A: fewer than 3 areas of lymphoid tissue are enlarged; with no anemia or thrombocytopenia<br>• Stage B: 3 or more areas of lymphoid tissue are enlarged; with no anemia or thrombocytopenia<br>• Stage C: anemia and/or thrombocytopenia are present |

(Modified from American Cancer Society, 2005)

### 1.1.2.3 Conventional Approaches to Leukemia Therapy

Nowadays, the three most common tactics for treatment of leukemias include chemotherapy, radiotherapy, and bone marrow or peripheral blood stem cell transplantation (SCT). Treatment options are based on the leukemia subtypes and disease stages. In chemotherapy, drugs are given in a vein, a muscle, into the cerebrospinal fluid (CSF), or taken by mouth to achieve remission by eliminating most of the normal and leukemic bone marrow (American Cancer Society, 2005). Combination cytotoxic chemotherapy with several drugs is often used over a period of time to enhance the cytotoxic effect and to prevent relapse. The chemotherapy regimen for AML usually includes cytarabine (ara-C) and an anthracycline drug such as daunorubicin or idarubicin; whereas the regime for ALL often involves cyclophosphamide, vincristine, dexamethasone (or prednisone), L-asparaginase, and doxorubicin (or daunorubicin) (American Cancer Society, 2005). In CML, the "Philadelphia chromosome" generates the abnormal *bcr-abl* gene and a constitutively activated Bcr-Abl protein. Since Bcr-Abl is the causative abnormality in CML, Imatinib mesylate, an orally available tyrosine kinase inhibitor, is applied to specifically block Bcr-Abl tyrosine kinase activity. Clinical trials have demonstrated that imatinib mesylate produces rapid responses in patients with all stages of CML (Duffy, 2003). For patients with CLL, they are usually treated with chlorambucil. Cyclophosphamide may be substituted if chlorambucil causes side effects. Combination therapy may sometimes be employed – the combined use of cyclophosphamide with doxorubicin and other drugs such as vincristine and prednisone (American Cancer Society, 2005).

In fact, chemotherapy is the double-edged sword of modern medicine.

Chemotherapeutic drugs, whatever form, is made up of toxins meant to non-specifically kill all actively dividing cells involving not just cancer cells but also normal fast growing cells in the bone marrow, hair follicles, and the lining of digestive and reproductive tracts (Hoffbrand *et al.*, 2001; American Cancer Society, 2005). As a result, chemotherapy can lead to serious side effects in patients. Reduced production of blood platelets results in easy bruising or bleeding, low white blood cell counts contributes to a higher risk of infection, and low red blood cell counts brings about anemia and tiredness. Moreover, some anticancer drugs can affect a patient's fertility; other common side effects include hair loss, loss of appetite, nausea and vomiting (American Cancer Society, 2005). Apart from the side effects, multidrug resistance (MDR) is also a major concern in cancer patients. Development of resistance towards chemotherapeutic drugs is associated with myriad mechanisms that diminish drug cytotoxicity. P-glycoprotein and multidrug resistance protein (MRP) are two members of the large family of ATP-binding cassette transporters that confer MDR in human cancer cells. P-glycoprotein, a membrane glycoprotein encoded by the *MDR1* gene, reduces the intracellular concentration of chemotherapeutic drugs by acting as a drug efflux pump. P-glycoprotein exports many types of drugs, including *Vinca* alkaloids, anthracyclines, paclitaxel, actinomycin D, and epipodophyllotoxins (Gouaze *et al.*, 2005).

In radiotherapy, high-energy rays or particles are employed to kill leukemia cells. For patients with acute leukemias, radiation may be applied when leukemia cells have spread to the brain and spinal fluid or to the testicles. For patients with chronic leukemias, radiation therapy is usually not the main treatment. However, it is used to shrink the enlarged internal organs, such as the spleen, to prevent them from pressing on other organs. Radiation therapy is also useful in treating pain due to bone damage

as a result of leukemic cell growth within the bone marrow. Moreover, radiation is given to several parts of the body prior to bone marrow or peripheral blood stem cell transplantation (American Cancer Society, 2005). Radiation attacks both the proliferating cancer cells as well as rapidly growing cells of normal tissues. The main short-term side effects of radiotherapy are sunburn-like skin changes in the treated area, fatigue, and reduced resistance to infection (American Cancer Society, 2005).

Stem cell transplantation (SCT) is regarded as a standard therapy that permits the use of high-dose chemotherapy and total body radiotherapy to kill all the "hidden" leukemia cells in patients' body, which result in destruction in their bone marrow. Patients can receive SCT to restore their bone marrow following chemotherapy and radiotherapy. For SCT, stems cells are collected from the bone marrow (known as bone marrow SCT) through bone marrow aspiration or from the bloodstream (known as peripheral blood SCT) in a process called apheresis (American Cancer Society, 2005). Post-transplantation therapies include drugs such as prednisone and cyclosporine that can weaken a patient's immune system to prevent it from rejecting the transplant. For the next few weeks patients are given supportive therapies such as intravenous nutrition, anti-bacterial and anti-fungal antibiotics, red blood cell transfusions, platelet transfusions, or other medications if necessary (American Cancer Society, 2005). In fact, such clinical procedures are very expensive and tricky in searching suitable major histocompatibility complex (MHC) compatible donors for patients receiving allogeneic SCT. Moreover, life-threatening graft-versus-host disease (GVDH) still represents a major complication of allogeneic SCT, which occurs when the immunocompetent donor T lymphocytes contained in the graft attack the patient's epithelial surfaces of the skin and mucous membranes, biliary ducts of the liver, and crypts of the intestinal tract, as a result of MHC disparities between the

donor and the immunosuppressed patient (Vargas-Diez *et al.*, 2005). The most disabling symptoms of GVHD are severe skin rashes and severe diarrhea. On the positive side, however, GVHD can also lead to graft-versus-leukemia activity (American Cancer Society, 2005). Leukemia cells remaining after the chemotherapy and radiotherapy will often be eradicated through immune rejection by the donor immune cells.

### 1.1.2.4 Novel Approaches to Leukemia Therapy

While chemotherapeutic drugs eradicate leukemia cells, they can also kill our rapidly growing cells as a result of adverse cytotoxicity. In addition, certain side effects of the drugs often damage human organs including the kidneys, liver, brain, heart, lungs, testes, and ovaries (American Cancer Society, 2005). A representative example is tumor lysis syndrome, a side effect caused by the rapid breakdown of leukemia cells during cancer treatment, constituting a major source of morbidity and mortality. Very often, these dying leukemia cells release substances into the bloodstream leading to acute renal failure (Lameire *et al.*, 2005). Many other undesirable side effects along with conventional chemotherapies also put forward the need to develop novel strategies for treatment of hematologic malignancies.

Leukemia results from disruption of the fine balance between proliferation and differentiation of hematopoietic stem cells and/or progenitor cells. Over the past decade, significant breakthroughs have been made to arrest the cell cycle, to provoke apoptosis, or to induce differentiation of leukemia cells by natural products (Fung *et al.*, 1997; Mak *et al.*, 2002; Lung *et al*, 2004). A paradigm is provided by the clinical development of a non-chemotherapeutic drug known as all-trans-retinoic acid (ATRA)

in the treatment of acute promyelocytic leukemia (APL), through induction of differentiation of APL cells into mature granulocytes (Huang *et al.*, 1988; Pandolfi, 2001). After remission of APL, further treatment usually consists of two or more courses of chemotherapy followed by maintenance with ATRA for at least one year. About seven out of ten patients are effectively cured with this approach (American Cancer Society, 2005). Other examples of naturally occurring anti-tumor compounds include 1,25 dihydroxycholecalciferol (vitamin D3), quercetin (flavonoid), biochanin A (isoflavonoid), epigallocatechin-3-gallate (EGCG, green tea catechin), trans-3,4',5-trihydroxystilbene (red wine resveratrol), and arsenic trioxide ($As_2O_3$). Among them, vitamin D3 and $As_2O_3$ are employed in clinical trials for hematologic malignancies (Miller and Waxman, 2002). While vitamin D3 had been reported to induce $G_0/G_1$ phase cell cycle arrest, differentiation, and apoptosis of human APL HL-60 and NB4 cells (Hisatake *et al.*, 2001), $As_2O_3$ also induced apoptosis in APL cells (Mak *et al.*, 2002) and triggered cellular differentiation of APL cells from patients who had relapsed (Soignet *et al.*, 1998). Similarly, quercetin also exhibited apoptosis-inducing effect on human APL HL-60 cells (Shen *et al.*, 2003), and biochanin A induced monocytic differentiation of murine myelomonocytic leukemia WEHI-3B JCS cells (Fung *et al*, 1997). Moreover, the green tea catechin EGCG inhibited the growth of leukemia cells by inducing differentiation of the human eosinophilc leukemia EoL-1 cells (Lung *et al.*, 2002). Resveratrol, a phytoalexin often found in skins of red grapes and red wines, inhibited the proliferation of human APL HL-60 cells through induction of apoptosis (Kang *et al.*, 2003).

Therefore, there is an increasing interest in the use of natural products as complementary and/or alternative medicines against leukemias in recent years (American Cancer Society, 2005). They are good candidates for cancer therapy not

only by providing low-dose chemotherapy in combination with conventional treatments, but also by assuaging side effects, alleviating drug resistance, and lessening recurrence of malignancies. Over the past two years, we have sought to address this issue by studying the anti-tumor activities of a kind of dietary fats, the conjugated linoleic acid (CLA), on myeloid leukemia cells. We have found a few distinct yet complementary mechanisms that CLA retards leukemic cell growth, which will be delineated in detail in chapters 3, 4 and 5 of this M.Phil. dissertation.

# 1.2 Conjugated Linoleic Acid

## 1.2.1 Introduction: Historical Development and Occurrence of Conjugated Linoleic Acid

In general, the term "conjugated linoleic acid" and its acronym "CLA" refers to a group of positional and geometric conjugated dienoic isomers of omega-6 essential fatty acid, linoleic acid (Pariza *et al.*, 2001). All *cis*- (Z-) and *trans*- (E-) isomeric combinations of CLA have been virtually identified in human diet, of which the most commonly occurring CLA isomer found in food is *cis*-9, *trans*-11 octadecadienoic acid (*cis*-9, *trans*-11 CLA), followed by *trans*-7, *cis*-9 CLA, *cis*-11, *trans*-13 CLA, *trans*-11, *cis*-13 CLA, *cis*-8, *trans*-10 CLA, *trans*-8, *cis*-10 CLA, and *trans*-10, *cis*-12 CLA (Kelly, 2001; Belury, 2003). Numerous fatty acids with conjugated double bonds appear naturally in edible fats derived from ruminant animals, for example milk fat and beef tallow (Pariza *et al.*, 2001). However, CLA is not found to exist in significant amount in plants. According to an examination on CLA content in milk from various herds of cows in New York State, CLA levels ranged from 2.4 to 18 mg CLA/g fat (Ma *et al.*, 1999).

Prior to 1987, scientific attention in CLA was largely confined to rumen microbiologists who studied the *cis*-9, *trans*-11 CLA isomer as an intermediate in the biohydrogenation of linoleic acid (Parodi, 1999). Significant breakthroughs have been made in the research of CLA when Ha *et al.* (1987) reported that CLA produced by base-catalyzed isomerization of linoleic acid was an effective inhibitor of benzo(a)pyrene-initiated mouse epidermal neoplasia. Since then, copious biological and physiological effects of CLA have been reported, which will be discussed in the following sections in detail.

### 1.2.2 Phytochemistry and Metabolism of Conjugated Linoleic Acid

#### 1.2.2.1 Chemical Structures of Conjugated Linoleic Acid Isomers

The biochemical nomenclature for linoleic acid designates as *cis*-9, *cis*-12 octadecadienoic acid (C18:2). Linoleic acid has an 18-carbon ("octa-deca") fatty acid backbone containing two double bonds ("di-en") at the 9 and 12 carbon atoms in a *cis*-isomeric configuration. This structural configuration results in two double bonds separated by two single bonds. CLA is formed when reactions shift the location of one or both double bonds of linoleic acid in such a way that the two double bonds are separated by one single bond. As a result, several dozen different CLA isomers are possible, depending on which double bonds are relocated and the resulting *cis*- or *trans*-isomeric reconfigurations (Kelly, 2001). Fig. 1.2 depicts the chemical structures of linoleic acid and several common CLA isomers. However, some commercial CLA preparations contain additional isomers with conjugated double bonds at positions 8 and 10, or 11 and 13 (Pariza *et al.*, 2001).

**Fig. 1.2: Chemical structures of linoleic acid and several common CLA isomers.**

### 1.2.2.2 Biosynthesis of Conjugated Linoleic Acid

CLA is found predominantly in products of ruminants and is formed as a result of incomplete microbial biohydrogenation (metabolism) of dietary unsaturated fatty acids in the rumen (Kelly and Bauman, 1996). The pathway by which linoleic acid is biohydrogenated to stearic acid is presented in Fig. 1.3. When biohydrogenation is not complete, CLA can escape the rumen and be absorbed from the digestive tract, thereby providing the mammary gland with a source of CLA which is found in milk fat (Kelly and Bauman, 1996). The endogenous rumen bacteria primarily responsible for the biohydrogenation are known as *Butyrivibrio fibrisolvens*, which have linoleic acid isomerase enzyme activity (Kelly, 2001). Although CLA is an intermediate in ruminal biohydrogenation of linoleic acid, the cow can also synthesize CLA in mammary gland from *trans* fatty acid (trans-11 18:1) with an enzyme called Δ9-desaturase (Griinari *et al.*, 2000). This enzyme inserts a *cis*-double bond at carbon 9 of the *trans*-11 fatty acid to form the *cis*-9, *trans*-11 CLA isomer. This biochemical pathway attributes to the majority of CLA found in products generated from milk fat of lactating cows (Kelly, 2001). Interestingly, adipose tissues of growing sheep and cattle have substantially greater amount of Δ9-desaturase, therefore adipose tissue seems to be the pivotal site of endogenous synthesis of *cis*-9, *trans*-11 CLA in growing ruminants; and mammary gland is the apparent site of endogenous synthesis of *cis*-9, *trans*-11 CLA in lactating ruminants (Bauman *et al.*, 1999). As a result of the isomerization and desaturation reactions in cows, *cis*-9, *trans*-11 CLA is thereby the principal CLA isomer in the human diet.

For the *trans*-10, *cis*-12 CLA isomer, it has been reported that *Propionibacter* can convert linoleic acid to *trans*-10, *cis*-12 CLA. Certain as-yet-unidentified rumen

bacteria also appear to possess this capability to form *trans*-10, *cis*-12 CLA in rumen digesta, in addition to *cis*-9, *trans*-11 and *trans*-9, *trans*-11 CLA (Pariza *et al.*, 2001). Although cow's milk is reported to contain *trans*-10, *cis*-12 CLA and *trans*-10 fatty acid, since mammals do not possess Δ12 desaturase, they could not subsequently convert *trans*-10 fatty acid into *trans*-10, *cis*-12 CLA. Accordingly, the *trans*-10, *cis*-12 CLA reported in milk fat would seem to originate solely from *trans*-10, *cis*-12 CLA that is absorbed from the gastrointestinal tract. In addition, the sources of other CLA isomers that occur naturally in milk fat are not known, but it is most likely that they are derived from bacterial metabolism in the rumen (Pariza *et al.*, 2001). In fact, CLA content of milk fat can be influenced by directly manipulating the type of dietary supplements fed to dairy cows. Supplementing cows' diet with polyunsaturated oils that contain either linoleic acid (e.g. corn oil or sunflower oil) or linolenic oil (e.g. fish oil) increases CLA content of milk fat substantially (Kelly, 2001). Furthermore, it has been reported that not just the quantities but also the ratios of CLA isomers produced in the rumen and subsequently found in beef fat may be affected by diet (Pariza *et al.*, 2001).

In humans, low concentrations of CLA are found in human blood and tissues (MacDonald, 2000). However, unlike ruminants, human production of CLA from free radical-mediated oxidation of linoleic acid does not appear to occur to any significant degree. In one experiment, feeding 16 g/day linoleic acid for six weeks resulted in no changes in plasma levels of CLA (Herbel *et al.*, 1998). In mice, tissue CLA levels decline steadily following the withdrawal of CLA from the diet. Since tissue levels of CLA in humans seem to be a direct reflection of dietary exposure to CLA, it is likely that a similar decline would result in humans subsequent to CLA withdrawal from the diet (Kelly, 2001). The primary isomer that builds up in human tissues subsequent to

milk fat intake is also the *cis*-9, *trans*-11 CLA.

Some bacterial species in the large intestine of monogastric animals, for example *Lactobacillus reuteri* from rat colon, also possess the capacity to convert linoleic acid into *cis*-9, *trans*-11 CLA through microbial metabolism (Pariza *et al.*, 2001). Although bacteria for linoleic acid conjugation were also detected in human intestinal tract, CLA synthesis from dietary linoleic acid was not observed. Emerging evidence suggests that the bacterial linoleic acid-conjugating activity is inhibited by glucose and other carbohydrates in rats. The total concentrations of glucose and total reducing carbohydrates found in cecum and colon contents of rats were sufficiently high to inhibit CLA synthesis *in vivo* (Kamlage *et al.*, 2000).

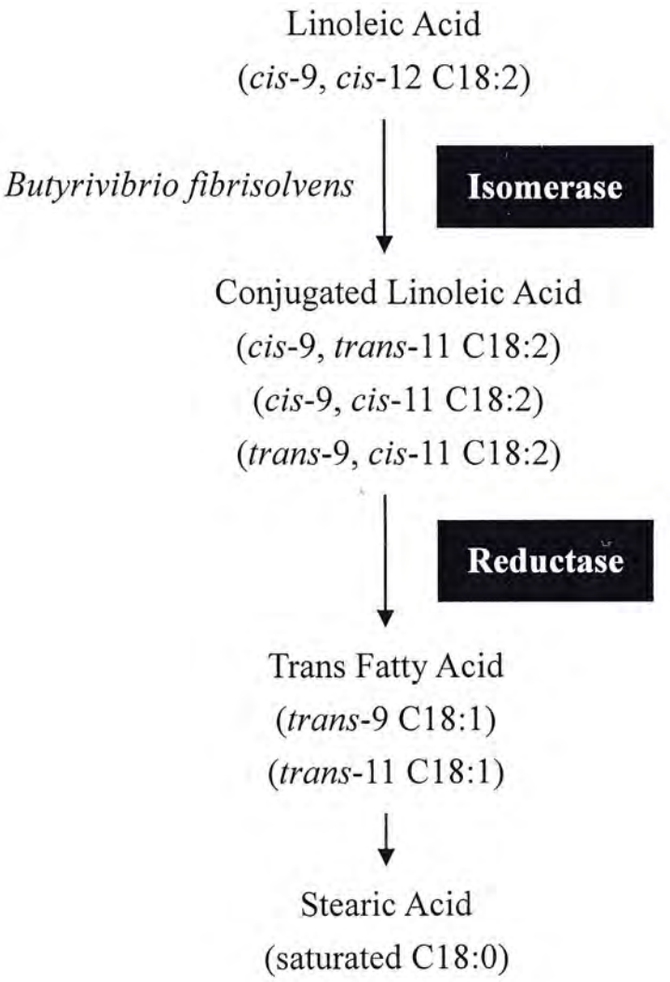



**Fig. 1.3: The pathway of biohydrogenation of linoleic acid to stearic acid by rumen microorganisms.**

(Modified from Kepler *et al.*, 1966; Kelly and Bauman, 1996)

### 1.2.2.3 Metabolism of Conjugated Linoleic Acid

Of 28 positional and geometrical isomers of CLA, the only CLA isomers that have been shown to be metabolized *in vivo* are *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA which can undergo Δ6 desaturation, elongation and further Δ5 desaturation, while maintaining the conjugated diene (CD) double bond structure. As a result, the two CLA isomers (CD18:2) form conjugated γ-linolenate (CD18:3; by introducing a double bond at position 6), conjugated eicosatrienoate (CD20:3; by adding two carbon atoms) and conjugated arachidonic acid (or conjugated eicosatetraenaote, CD20:4; by introducing a double bond at position 5) (Banni, 2002). Indeed, emerging evidence indicates that all these elongated and desaturated metabolites have been identified in the liver and mammary tissue of rats and adipose tissue and sera of humans (Belury, 2002). Moreover, other metabolites with 16 carbon atoms, possibly derived from peroxisomal β-oxidation of CLA and its metabolites, have been detected. CD16:2 is most probably derived from CLA, whereas CD16:3 is most probably derived from CD20:4 (theoretically, CD16:3 could also be derived from CD18:3 and CD20:3) (Banni, 2002). In fact, it has been reported that linoleic acid is metabolized to the same extent, to form CD18:3, as CLA when compared in an enzymatic study using a hepatic isolate of Δ6 desaturase enzyme (Belury, 2002). The schemes of CLA as well as linoleic acid metabolism are depicted in Fig. 1.4.

Metabolism of CLA and its metabolites in peroxisomes suggests that they might be capable of activating peroxisome proliferator-activated receptors (PPAR). CLA has been reported to ligate and activate PPAR-α, and to induce key enzymes of peroxisomal β-oxidation (Moya-Camarena *et al.*, 1999). Interestingly, conjugated arachidonic acid derived from *trans*-10 or *cis*-10 CLA possesses double bonds in

positions Δ5,8,12,14, which is similar to a PPAR-γ ligand, raising the possibility that CD20:4 Δ5,8,12,14 may also be a PPAR-γ ligand (Banni, 2002). Nevertheless, the role of CLA metabolites in modulating tissue responses such as adipose tissue mass, glucose sensitivity, carcinogenesis, and/or tumor formation is pending further investigation. Yet the physiological activities of CLA metabolites are hampered by the lack of availability of purified metabolites (e.g. CD18:3, CD20:3, and CD20:4) for study in cell culture and *in vivo* feeding experiments (Belury, 2002).

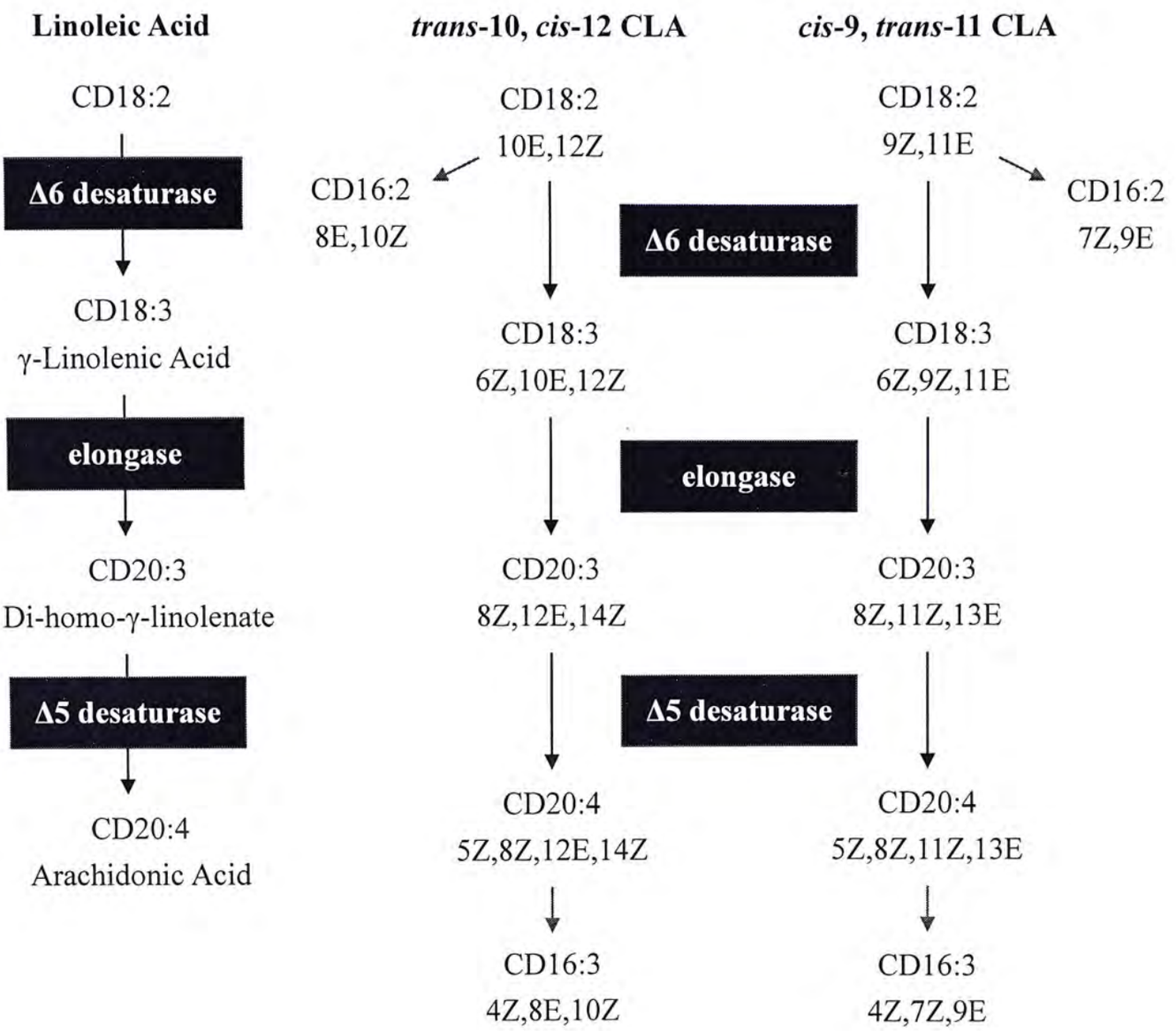



**Fig. 1.4. Schemes of CLA and linoleic acid metabolism**

Key: Z – *cis* configuration;
E – *trans* configuration;
Red arrow – peroxisomal β-oxidation.

(Modified from Belury, 2002; Banni, 2002).

### 1.2.2.4 Mode of Entry and Tissue Incorporation of Conjugated Linoleic Acid

CLA can be fed as free fatty acid or as triglyceride. Since it is lipid-soluble in nature, CLA is freely incorporated into the membrane phospholipids such as phosphatidylcholine and cholesteryl esters (Kelly, 2001). The majority of commercially available CLA is in the form of free fatty acids but not the triglyceride-bound CLA as found in food. It is not currently known whether the pharmacokinetics of CLA preparations in humans is influenced by feeding CLA as free fatty acids or as triglyceride-bound preparation (Kelly, 2001). Importantly, the accumulation of CLA isomers and several elongated, desaturated, and β-oxidation metabolites have been found in tissues of animals fed diets with CLA (Belury, 2002). The incorporation of CLA in humans is tissue-dependent, with adipose and lung tissues comprising the highest CLA concentrations (Kelly, 2001). However, the incorporations of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers into both plasma lipids (e.g. phosphatidylcholine and cholesteryl esters) and peripheral blood mononuclear cell lipids were found to be relatively low (Burdge *et al.*, 2004).

### 1.2.2.5 Toxicology of Conjugated Linoleic Acid

Rat toxicity data indicated that CLA intake as 1.5 percent of the diet for 36 weeks results in no histopathological damage to organs and no hematological abnormalities (Scimeca, 1998). In order to support the safety-in-use of Clarinol™ G80, a product with approximately equal proportions of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA which account for 75% of the total fatty acid content, two *in vitro* mutagenicity assays and a 90-day repeat-dose oral rat toxicity study were performed. The results showed that Clarinol™ G80 was non-mutagenic. In the 90-day toxicity

study, Clarinol™ G80 could lead to hepatocellular hypertrophy in female rats (but not the male ones) receiving the highest dose level (15% w/w). Because there were no changes in clinical chemistry and histology of the liver, such hepatocellular hypertrophy was not considered as adverse treatment-related effect and was regarded as an adaptive effect as it was reversible upon withdrawal of the test material. In addition, an increase in plasma insulin levels was also observed in these female rats but there was no effect on plasma glucose levels. Therefore, it has been concluded that a "No Observed Adverse Effect Level" (NOAEL) of 5% Clarinol™ G80, which is equivalent to 2433 mg/kg bw/day for male and 2728 mg/kg bw/day female rats, was identified in the study (O'Hagan and Menzel, 2003). Nonetheless, the effects of chronic use of CLA appear to be variable among animal species, genotype and sex (O'Hagan and Menzel, 2003). For instance, CLA at 1% of the diet has resulted in hepatomegaly in some mice (Kelly, 2001).

Other studies also concluded that CLA did not produce adverse effects in dogs and pigs even when fed at 5% in the diet (Pariza, 2004). Human clinical trial data on Clarinol™ G80 and other mixed isomer preparations of CLA have shown no effect on insulin and glucose levels (O'Hagan and Menzel, 2003). Moreover, in the published human clinical trials no effect on liver function has been reported. Aspartate aminotransferase (ALAT), alanine aminotransferase (ASAT), and γ-glutamyltransferase (γ-GT) levels were not affected by treatment with 1.7-6.8g/day CLA for a period of 12 weeks in both male and female volunteers (Blankson *et al.*, 2000). Adverse effects reported after CLA administration in human subjects may include gastrointestinal complaints and fatigue (Blankson *et al.*, 2000). In fact, there are contradictions concerning the effects of CLA on insulin and glucose homeostasis in man with recent reports indicating that CLA increases insulin level in plasma while

others show no effect on glucose level (Wahle *et al.*, 2004). Clearly, the documentations of adverse effects of CLA on health are in the minority but they need to be critically appraised before the health benefits of CLA can be arrived at.

### 1.2.3 Physiological Activities of Conjugated Linoleic Acid: Reported Health Benefits

Over the past two decades many health benefits (these will be discussed in sections 1.2.3.1 through 1.2.3.7) have been attributed to CLA (as small as 0.5% of diet) in experimental animal models including its actions on conditions such as obesity, atherosclerosis, carcinogenesis, tumor formation, and to delay the onset of diabetes and enhance our immune system (Belury, 2002). The overwhelming number of beneficial reports for CLA on the basis of studies with cells *in vitro*, with animal models of disease, and with human volunteers *in vivo* are very encouraging and warrant critical appraisal.

Owing to the high cost and/or lack of availability, very few studies conducted *in vivo* have used highly purified isomers or naturally extracted CLA oil (Belury, 2002). Therefore, the majority of research to date has been conducted on a synthetic mixture of CLA isomers, in which the *cis*-9, *trans*-11 CLA and the *trans*-10, *cis*-12 CLA are the predominant isomers constituting approximately 85 to 90 percent. These two isomers are usually represented in about equal quantities, with ten other minor CLA isomers representing the remaining 10 to 15 percent of the mixture (Kelly, 2001). Interestingly, the major effects of CLA are largely, but not exclusively, observed in mice treating with a mixture of CLA isomers or the *trans*-10, *cis*-12 but not the *cis*-9, *trans*-11 CLA (Pariza *et al*, 2001; Wahle *et al.*, 2004). It is also likely that some

effects are induced and/or enhanced by various CLA isomers acting synergistically (Pariza *et al*, 2001). However, their effective dosages and duration have not been elucidated *in vivo*.

### 1.2.3.1 Anti-adipogenesis

It has been suggested that *trans*-10, *cis*-12 CLA is the bioactive isomer of CLA that reduces adiposity with increased lean body mass (Pariza *et al*, 2001; Rainer and Heiss, 2004). The change in body composition is partly associated with a reduction in fatty acid uptake by adipocytes, the principal sites of fat storage (Azain *et al*., 2000; Choi *et al*., 2000; Xu *et al*., 2003). The reduction in lipid uptake by adipocytes, in turn, appears to be due to inhibitory effects of CLA on the enzymatic activities of stearoyl-CoA desaturase (SCD) (Choi *et al*., 2000; Park *et al*., 2000) and lipoprotein lipase (LPL) (Pariza *et al*, 2001; Xu *et al*., 2003). In addition, it had been reported that apoptosis was triggered in adipose tissue of mice fed diet at 1% CLA, an isomer mixture with about 40% *trans*-10, *cis*-12 CLA, supporting that *trans*-10, *cis*-12 CLA was able to induce apoptosis in cultured 3T3-L1 mouse adipocytes (Tsuboyama-Kasaoka *et al*., 2000; Pariza *et al*, 2001). By contrast, other groups reported that feeding diet supplemented with 0.25 or 0.5% CLA for 5 weeks to Sprague-Dawley rats reduced adipocyte cell size but not number (Azain *et al*., 2000; Xu *et al*., 2003). Hence the CLA-induced apoptosis in adipose tissue may be species dependent. Such an effect was observed in mice but not in the rats.

The induction of adipocyte differentiation is also another mechanism responsible for changes in body composition by CLA. Induction of markers of adipocyte differentiation by CLA was first shown *in vivo* in male Zucker diabetic fatty (ZDF,

fa/fa) rats fed with 1.5% CLA for 2 weeks. In this study the adipocyte lipid-binding protein ap2, a marker of adipocyte differentiation, was increased approximately five fold relative to rats fed diet without CLA (Houseknecht *et al.*, 1998). At present, there is little support for the hypothesis that CLA might enhance lipolysis. In particular, CLA (specifically the *trans*-10, *cis*-12 CLA) would block body fat gain, but not necessarily reduce body fat level which had accumulated prior to CLA administration (Pariza *et al*, 2001; Xu *et al.*, 2003). Nevertheless, one should not assume that CLA (and *trans*-10, *cis*-12 CLA) will necessarily exhibit a single defined set of effects on all adipocytes irrespective of other biological considerations. It is possible that the effects of CLA on a given adipocyte will depend, at least in part, on the location, microenvironment and physiological function of that adipocyte (Pariza *et al.*, 2001). For instance, the CLA-induced reduction in adiposity may under certain circumstances be overwhelmed by a diet that is excessively high in fat. Pariza *et al.* (2001) had pointed out that more researches should be considered in light of the interplay between adipocytes (fat storage) and skeletal muscle cells (fat combustion). However, the possible effects of CLA on skeletal muscle are less understood than that of adipocytes.

Despite the evidence that CLA improves lipid profile in animal models, studies using human subjects are currently ambiguous to advocate whether CLA supplementation has a significant effect on human body composition (Kelly, 2001; Rainer and Heiss, 2004). Certain studies found no significant changes in body composition among obese subjects given CLA (Zambell *et al.*, 2000; Kamphuis *et al.*, 2003). By contrast, other studies reported a trend toward decreased body weights and body mass index (BMI) among subjects receiving CLA (Blankson *et al.*, 2000; Pariza *et al*, 2001). Additionally, it was also reported that the plasma levels of *trans*-10,

*cis*-12 CLA were inversely associated with body weight and serum leptin, a hormone known to regulate fat intake, in subjects with type 2 diabetes mellitus (DM) (Belury *et al.*, 2003), and *trans*-10, *cis*-12 CLA was able to prevent accumulation of triglyceride in primary cultures of human differentiating preadipocytes (Brown and McIntosh, 2003).

### 1.2.3.2 Anti-diabetogenesis

Central to all of the risk factors leading to type 2 DM is obesity. There is increasing evidence showing that CLA is able to delay the onset of type 2 DM. In a male Zucker diabetic fatty (ZDF) rat model, rats were fed with semi-purified diets containing no CLA (control), 1.5% CLA, or the anti-diabetic drug, troglitazone (0.02%) for two weeks. Rats fed with the diet containing CLA or troglitazone exhibited significant reduction in fasting glucose, insulinemia, triglyceridemia, free fatty acid levels, and leptinemia compared with control rats (Belury, 2002). While CLA reduces fasting insulin in diabetic animals, it modestly elevates fasting serum insulin in non-diabetic mice and humans (Tsuboyama-Kasaoka *et al.*, 2000; Medina *et al.*, 2000). Since fasting insulin may be used as a surrogate marker for insulin resistance, these data suggest that CLA reduces insulin sensitivity under a normoglycemic state. In one experiment, after long-term feeding (8 months) of a CLA-diet, an induction of insulin resistance was observed in C57BL/6J male mice (Tsuboyama-Kasaoka *et al.*, 2000). The impact and significance of CLA-induced insulin resistance for people who are normoglycemic is yet unknown.

Because CLA is capable of delaying the onset of DM in the ZDF rat model, CLA as an aid in the management of type 2 DM in humans was examined in a

double-blinded study. Subjects with type 2 DM were randomized into one of two groups receiving either a supplement containing a mixture of CLA isomers (8.0 g daily, n = 12) or a supplement containing linoleic acid as a control (8.0 g safflower oil daily, n = 9) for 8 weeks. The isomers of CLA in the mixtures were primarily *cis*-9, *trans*-11 CLA (approximately 37%) and *trans*-10, *cis*-12 CLA (approximately 39%) in free fatty acid form. It was found that supplementation with CLA for 8 weeks could be associated with favorable alterations of several metabolic parameters in subjects with type 2 DM. Moreover, the plasma levels of CLA were inversely associated with body weight and serum levels of leptin, a hormone known to regulate fat intake (Belury *et al.*, 2003).

### 1.2.3.3 Anti-atherosclerosis

There is a large and growing body of evidence indicating that CLA reduces atherosclerotic plaque formation in rabbits and hamsters. In one experiment, when CLA (0.5 g daily) was added to a hypercholesterolemic diet for rabbits, both the serum triglyceride and the low density lipoprotein (LDL) cholesterol levels were significantly reduced relative to rabbits fed with a diet devoid of CLA after 12 weeks. Moreover, the aortas of rabbits fed with the CLA-containing diet showed less atherosclerotic plaque formation (Lee *et al.*, 1994). The ability of CLA to reduce aortic plaque formation could be resulted from changes in LDL oxidative susceptibility (Belury, 2002). In another study, hamsters were fed with a diet supplemented with or without CLA to induce hypercholesterolemia. The diet with 1% CLA reduced plasma total cholesterol, non-high density lipoprotein cholesterol, and early aortic atherosclerosis compared to a diet devoid of CLA (Wilson *et al.*, 2000). On the contrary, CLA was shown to induce the formation of aortic fatty streaks in

C57BL/6 mice fed with an atherogenic diet (Munday *et al.*, 1999). Because of the differential effects of CLA in various animal models, further work is needed to elucidate the mechanisms by which CLA can cause the reduction of atherosclerotic plaque formation in humans.

### 1.2.3.4 Anti-carcinogenesis

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are probably the major omega-3 fatty acids in fish oil responsible for cancer suppression. However, the amount of fish oil needed to elicit this response usually exceeds 10% in human diet. By contrast, CLA concentration as low as 0.1% was adequate to produce a significant reduction in mammary-tumor yield in rats challenged with a low dose of carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA). Therefore, CLA together with various other anti-carcinogens may provide chemoprevention against cancer at concentrations close to human consumption levels (Macdonald. 2000). In fact, in a publication of the National Academy of Science, Carcinogens and Anticarcinogens in the Human Diet, it was concluded that "... conjugated linoleic acid (CLA) is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals." (National Research Council, 1996).

The biological activity of CLA was discovered as a consequence of its inhibitory effects on chemically-induced epidermal carcinogenesis in mice (Ha *et al.*, 1987) and subsequent research using rat carcinogenesis models (Liew *et al.*, 1995; Pariza *et al.*, 2001). CLA was also found to inhibit chemically-induced mammary carcinogenesis in a dose-dependent manner regardless of whether it was fed as a free fatty acid or

triglyceride (Ip *et al.*, 1996). The *cis*-9, *trans*-11 CLA isomer by itself has been shown to effectively reduce chemically-induced rat mammary neoplasia (Pariza *et al.*, 2001). Nevertheless, there is also evidence for a synergistic interaction between *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in inhibiting mammary carcinogenesis in rats (Belury, 2002). The biochemical mechanism whereby CLA inhibits carcinogenesis may involve effects on the metabolism of linoleic acid (because CLA and linoleic acid share the same enzyme system) and vitamin A, therefore it is possible that both isomers might affect linoleic acid metabolism as well as exerting individual biochemical effects to modulate carcinogenesis (Pariza *et al.*, 2001).

Apart from linoleic acid metabolism, efforts have been made to elucidate the anti-carcinogenic mechanisms of CLA by determining its effects on different stages of carcinogenesis – initiation, promotion, and progression. In one study a CLA-containing lipid fraction extracted from fried ground beef was topically applied to mouse skin before initiation with DMBA. Tumor yield, interpreted as average number of tumors per mouse, after 16 weeks of promotion with 12-0-tetradecanoylphorbol-13-acetate (TPA) was shown to be inhibited by approximately 45% (Ha *et al.*, 1987). As an anti-initiator, CLA may modulate events such as free radical-induced oxidation, carcinogen metabolism, and/or carcinogen-DNA adduct formation in some tumor models (Belury, 2002). In addition to its anti-initiator activity, CLA was also shown to inhibit tumor promotion. The promotion stage involves clonal expansion of initiated cells, as a result of increased cell proliferation, reduced apoptosis, and/or dysregulated differentiation, to form a benign tumor (Belury, 2002). In a subsequent study, rats that were fed with diets containing 1% CLA following initiation with methylnitrosourea exhibited reduced proliferation and thereby reduced the density of the terminal end bud as well as

lobuloalveolar bud structures of mammary epithelium (Thompson *et al.*, 1997). Importantly, the terminal end bud is the site of tumor formation for both rat and human breast cancer. More recently, CLA-induced anti-proliferation of the terminal end bud was found to be accompanied by reduced levels of two cyclins, cyclin D1 and cyclin A (Ip *et al.*, 2001). These data suggest that CLA modulates molecular signaling events that regulate the cell cycle, ultimately regulating cell proliferation. Nonetheless, it has been demonstrated that the ability of CLA to inhibit cell growth may be tissue-specific (Belury, 2002). In other tissue models, the mechanistic role of CLA in modulating carcinogenesis involves induction of apoptosis as well as differentiation. A large growing body of evidence suggests that CLA can induce apoptosis in numerous tissues including mammary, liver, and adipose tissues (Tsuboyama-Kasaoka *et al.*, 2000; Belury, 2002). In these studies, CLA-induced apoptosis was correlated with a reduction of bcl-2, a signaling protein known to repress apoptosis. Furthermore, CLA can induce differentiation of the adipose tissue, suggesting that it may inhibit carcinogenesis through modulating tissue differentiation (Belury, 2002).

It is critical to apprehend how CLA modulates metastasis of the malignant tumors which contribute the leading cause of morbidity and mortality in cancer patients. In recent years CLA has also been shown to inhibit the progression stage of carcinogenesis. At least one study revealed that CLA (0.5-1%) inhibited proliferation of the transplanted mammary cancer cells to form secondary tumors in mice (Belury, 2002). Moreover, a Chinese group of researchers reported that the *cis*-9, *trans*-11 CLA isomer hampered the invasion of human gastric carcinoma cells via inhibition of the metastasis-associated gene expression (Yang *et al.*, 2003). Therefore, the possibility that a higher intake of CLA might reduce the risk of metastasis cannot be ruled out. However, more studies should be carried out to substantiate the protective

effect of CLA on the risk of metastasis.

### 1.2.3.5 Anti-tumor Activity

The anti-tumor potential of CLA has received a great deal of research attention in both *in vitro* and *in vivo* animal models. A growing body of evidence has demonstrated the *in vitro* anti-tumor activity of CLA. CLA suppressed tumor growth and served as a cytotoxic mediator to a variety of human cancer cell lines including hepatoma cells, HepG2 (Igarashi and Miyazawa, 2001; Yu-Poth *et al.*, 2003), lung adenocarcinoma cells, A-427, SK-LU-1, and A549 (Schonberg and Krokan, 1995), gastric carcinoma cells, SGC-7901 (Liu *et al.*, 2002; Chen *et al.*, 2003), colon carcinoma cells, HT-29, HCT116, and SW480 (Cho *et al.*, 2003; Kemp *et al.*, 2003; Lim *et al.*, 2005), colorectal cancer cells, MIP-101 (Palombo *et al.*, 2002), prostate carcinoma cells, LNCaP, and PC-3 (Song *et al.*, 2004; Ochoa *et al.*, 2004), and bladder cancer cells, TSU-Pr1 (Oh *et al.*, 2003). In addition, CLA also inhibited proliferation of estrogen receptor-positive breast cancer MCF-7 cells, and induced apoptosis of estrogen receptor-negative breast cancer MDA-MB-231 cells (Durgam *et al.*, 1997; Chujo *et al.*, 2003; Miglietta *et al.*, 2005). In these studies, the biochemical mechanisms of CLA-induced anti-tumor activity involved inhibition of tumor cell proliferation, modulation of tumor cell cycle progression, as well as induction of apoptosis and/or tumor cell differentiation. In fact, CLA not only demonstrated *in vitro* anti-tumor activity but also *in vivo* growth- and metastasis-inhibitory activities. The majority of animal tumor research has focused on the effects of CLA on mammary cancer (Kelly, 2001). In one study, severe combined immunodeficient (SCID) mice were fed with 1% CLA for two weeks before subcutaneous injection of

human breast adenocarcinoma MDA-MB468 cells, and 1% CLA administration was continued throughout the study period. It was found that CLA inhibited local tumor growth and tumor metastasis to lungs, peripheral blood, and bone marrow (Visonneau *et al.*, 1997), suggesting that dietary CLA can block the local growth and systemic spread of the human breast cancer cells via mechanisms that are independent of the host's immune system.

Unlike CLA, however, linoleic acid showed no growth-inhibitory activity against tumor cells *in vitro* or even stimulated the growth of some cancer cell lines (Banni *et al.*, 1999). It had been reported that incubation with linoleic acid resulted in a 25% increase in cell proliferation (Kelly, 2001). Moreover, the effects of CLA on leukemia cells have not been thoroughly studied.

### 1.2.3.6 Effects of Conjugated Linoleic Acid on Lipid Metabolism

Of the two predominant isomers of CLA, *cis*-9, *trans*-11 CLA accumulates to a greater extent than *trans*-10, *cis*-12 CLA in phospholipids of liver, skin, and bone tissues in animal models (Belury, 2002). The higher level of *cis*-9, *trans*-11 CLA may be owing to its preferred tissue incorporation and/or due to the *trans*-10, *cis*-12 CLA isomer has a more rapid metabolism (Belury, 2002). Nevertheless, CLA exhibits several effects on hepatic lipid metabolism, for instances, a mixture of CLA isomers (in which *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are the predominant isomers) reduced secretion of apolipoprotein B in cultured human hepatoma Hep-G2 cells and the *trans*-10, *cis*-12 CLA isomer inhibited the expression and activity of hepatic stearoyl-CoA desaturase (SCD) (Pariza *et al.*, 2001). In addition, *trans*-10, *cis*-12 CLA was found to be more effective than *cis*-9, *trans*-11 CLA on repressing

triacylglyceride secretion *in vitro* (Lin *et al.*, 2001).

#### 1.2.3.6.1 Actions on Phospholipids by Conjugated Linoleic Acid

Three theories have been reported by Belury (2002) to explain how CLA can affect the physiological activities including adiposity, diabetes, carcinogenesis, and immunity. Firstly, CLA can modulate the accumulation of arachidonic acid in phospholipids, resulting in a decreased pool of arachidonic acid and thereby the downstream eicosanoid products (Fig. 1.5). Secondly, CLA can modulate the expression, at both gene and protein levels, and/or activity of the cyclooxygenase-1 (a constitutive enzyme) and/or the cyclooxygenase-2 (an inducible enzyme) so as to reduce the production of eicosanoid products. While CLA is regarded as an anti-carcinogen, arachidonic acid may be a procarcinogen by causing subsequent production of prostaglandins (Iwakiri *et al.*, 2002). Iwakiri and coworkers showed that CLA decreased the concentration of prostaglandin $E_2$ by suppressing the transcription of cyclooxygenase-2 in macrophages (Iwakiri *et al.*, 2002; Yu *et al.*, 2002). Thirdly, it has been proposed that CLA or its elongated and desaturated products (e.g. conjugated arachidonic acid as shown in section 1.2.2.4 and Fig. 1.5) may act as substrates or antagonists for cyclooxygenases, and ultimately reducing the availability of enzymes for arachidonic acid. More likely, CLA acts antagonistically to repress the activity of cyclooxygenase in phospholipids by the formation and accumulation of the arachidonate analogue of CLA, the conjugated arachidonic acid (or conjugated eicosatetraenaote, CD24:4).

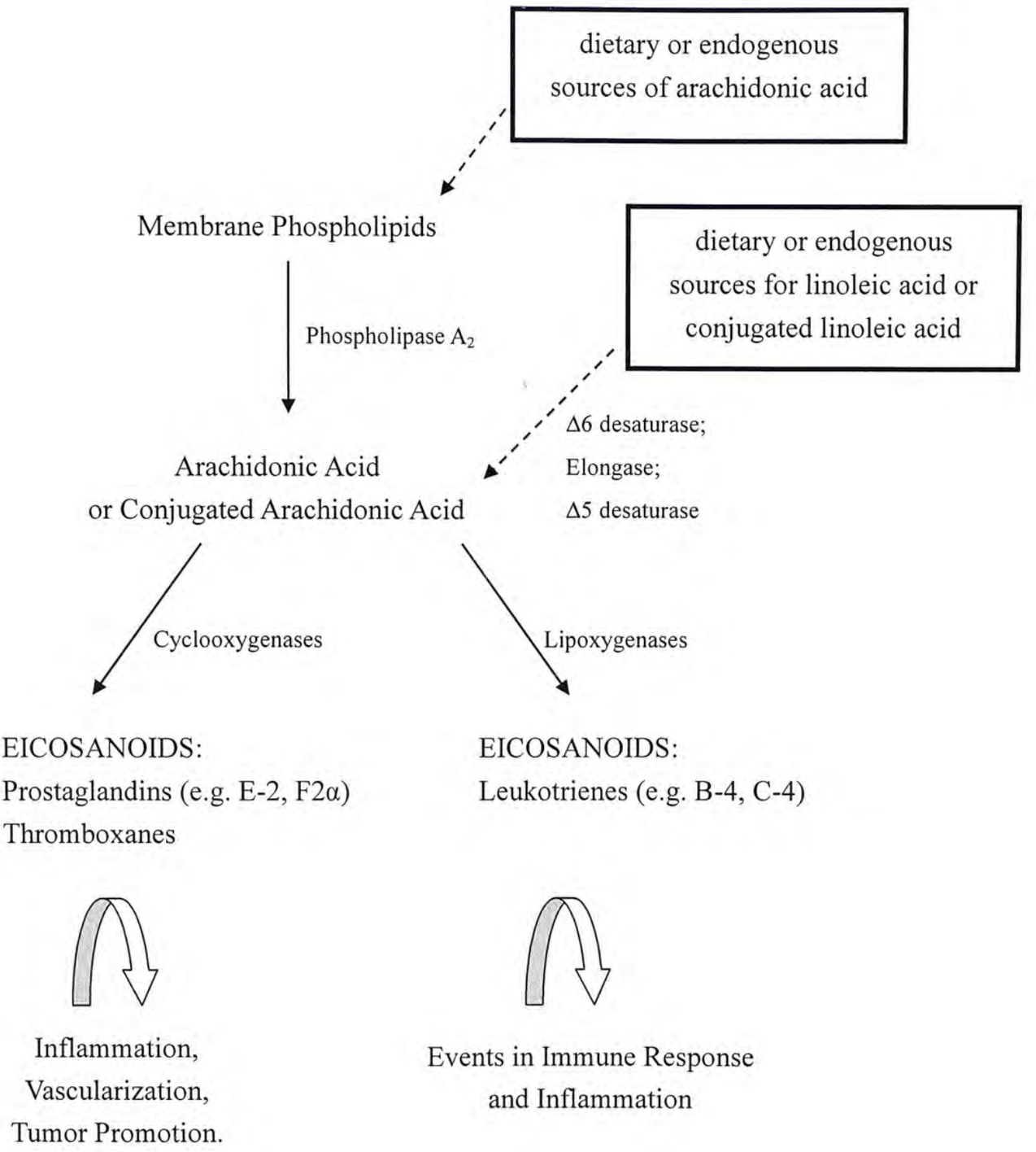



**Fig. 1.5: Schematic pathways for the synthesis of eicosanoids from arachidonic acids.**

(Modified from Belury, 2002)

### 1.2.3.6.2 Conjugated Linoleic Acid as a Ligand for the PPAR System

Until recently, the role of CLA on lipid metabolism in the liver and extra-hepatic tissues is basically unknown. CLA modulates lipid metabolism, in part, by activating a group of nuclear transcription factors, the peroxisome proliferator-activated receptors (PPAR) (Belury, 2002). More recently, new evidence demonstrates that activators of PPARγ are protective against cancers of the colon, prostate, and mammary gland (Sporn *et al.*, 2001). Therefore, it is feasible that some of the CLA-induced physiological activities are PPARγ-dependent (Belury, 2002). In fact, the PPARγ isoform is present mainly in the extra-hepatic tissues including adipose, colon, prostate, and mammary gland (Belury, 2002). In addition, CLA may increase the level of PPARγ, and PPARγ2 is found to be one of several transcription factors that are responsible for adipose tissue differentiation (Belury, 2002).

### 1.2.3.7 Immunomodulation

More recently, dietary CLA has been documented to offer beneficial effects to animals on inflammation-induced growth suppression, endotoxin-induced anorexia, mucosal damage and growth failure in experimental colitis, and antigen-induced type 1 hypersensitivity response (Field and Schley, 2004). The two predominant isomers of CLA appear to have similar effects on immune function (Wahle *et al*, 2004). In addition, CLA increased immunoglobulin production in rat spleen lymphocytes (Yamasaki *et al.*, 2000) and, on the other hand, decreased the production of mediators of inflammation, such as prostaglandin $E_2$, tumor necrosis factor-α (TNF-α), and nitric oxide, in macrophages by reducing the interferon-γ (IFNγ)-induced mRNA expression of the inducible cyclooxygenase 2, inducible nitric oxide synthase (iNOS), and TNF-α

(Yu *et al.*, 2002). Interestingly, it has been found that the effects of CLA on cellular immunity sustained for some time beyond the period of dietary supplementation (Field and Schley, 2004).

In a recent double-blinded, randomized, reference-controlled study, the roles of CLA (3 g/day, with 1:1 *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers) on immune functions of healthy human volunteers (n=28) of both sexes aged 25 to 50 were investigated (Song *et al.*, 2005). Subjects were arbitrarily divided into two groups, the reference group received high oleic sunflower oil whereas the treatment group was provided with a total 3 g CLA per day in 6 soft gel capsules for 12 weeks, which was followed by a 12-week washout period. Afterwards, blood samples were analyzed for the changes in levels of immunoglobulins (IgA, IgM, and IgE) as well as cytokines (TNFα, IL-1β, and IL-10). The results showed that the plasma levels of IgA and IgM were increased while the allergy-related IgE levels were decreased. In addition, CLA also reduced the levels of the proinflammatory cytokines, TNF-α and IL-1β, yet enhanced the levels of the anti-inflammatory cytokine, IL-10 (Song *et al.*, 2005). Nevertheless, this is the first human study to demonstrate that CLA can beneficially affect immune function, in part, by modulating mediators of immunity in the healthy volunteers. It had been previously reported that pharmacological PPARγ agonists inhibited the peripheral blood mononuclear cell synthesis of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) at the level of mRNA expression (Wahle *et al*, 2004). Since CLA is a known ligand for PPAR, this suggests that the observed reduction in proinflammatory cytokines with CLA in the human volunteer study of Song *et al.* could be due to PPAR activation (Wahle *et al*, 2004).

Current understanding of the cellular mechanisms of immunomodulation by

CLA is incomplete. Initial studies demonstrated that the levels of leukotriene-B4 and leukotriene-C4 in spleen and lungs were reduced in rats fed with 1% CLA in diet (Sugano *et al.*, 1998). Belury (2002) has postulated that CLA can modulate several events in immunity by regulating eicosanoid formation. Arachidonic acid-derived eicosanoids, from both cyclooxygenase and lipoxygenase pathways, are produced by various types of immune cells and are thought to regulate inflammation and cytokine synthesis (Belury 2002). However, in humans supplemented with CLA (3.9 g/day) for 93 days, no apparent changes were observed in the levels of eicosanoids including prostaglandin $E_2$ and leukotriene B-4 (Kelley *et al*, 2001). The discrepancy of data between different models of immune function suggests that the role of CLA in immunity awaits further investigations.

## 1.3 Aims and Scopes of This Investigation

Conjugated linoleic acid (CLA) is a fatty acid that is widely distributed in human diets and exhibit numerous beneficial physiological effects in various animal models and in humans, as described in Section 1.2.3. Until recently, the anti-tumor activities and action mechanisms of CLA on myeloid leukemia cells remain elusive. Moreover, their pro-apoptotic as well as differentiation-inducing activities on myeloid leukemia cells have not yet been studied. Therefore, the aim of my project is to investigate the anti-tumor activity of CLA against myeloid leukemia, and attempts were made to elucidate the cellular and molecular mechanisms governing their anti-tumor activity.

In the present study, the anti-proliferative effect of a mixture of CLA isomers and four of its individual isomers on the murine myelomonocytic leukemia cell line, WEHI-3B JCS, was compared. Four other leukemia cell lines of mouse and human origins, including M1, HL-60, NB4, and K-562, and a human lymphoma cell line, U-937, were used to demonstrate that the growth-inhibitory effect of CLA is not restricted only to a single leukemia cell line. The kinetics and reversibility of CLA with respect to their anti-proliferative effect on WEHI-3B JCS cells were assessed. The anti-proliferative and cytotoxic activities of CLA were evaluated by the colorimetric MTT assay or the $^{3}$H-thymidine incorporation assay and trypan blue exclusion assay, respectively. In addition, the cell cycle kinetics and cell cycle-regulatory gene expression following CLA treatment on WEHI-3B JCS cells were assessed by flow cytometry and RT-PCR, respectively. Moreover, the ability of CLA to induce apoptosis in myeloid leukemia cells was measured by the DNA fragmentation assay and semi-quantified by an ELISA kit. The expression of apoptosis-related genes was analyzed by RT-PCR while the expression of their protein

products was determined by Western blotting. Apart from determining the gene and protein expressions, the induction of caspase activities and mitochondrial membrane depolarization were also measured to elucidate the possible apoptotic pathway(s) induced by CLA. Furthermore, the effect of CLA on the *in vivo* tumorigenicity of WEHI-3B JCS cells was also studied in syngeneic BALB/c mice.

The differentiation-inducing activity of CLA on WEHI-3B JCS cells was assessed by a number of criteria, including their morphological, phenotypic, and functional alterations following CLA treatment. The morphological changes were studied by staining of cytocentrifuge preparations and by flow cytometry. The phenotypic changes were assessed by antibody staining of surface differentiation markers and measured by flow cytometry. Functional changes were evaluated by the induction of monocytic serine esterase activity and endocytic activity following exposure of the WEHI-3B JCS cells to CLA. Finally, the expression of differentiation-related cytokine genes was analyzed by RT-PCR.

# CHAPTER 2

# MATERIALS AND METHODS

# 2.1 Materials

## 2.1.1 Animals

Inbred female BALB/c (H-$2^d$) mice aged 6-8 weeks old were bred at the University Laboratory Animal Services Centre of The Chinese University of Hong Kong under a specific pathogen-free condition. They were fed with animal diet (Chow 5001, Rodent Laboratory) and tap water.

## 2.1.2 Cell Lines

### 1) WEHI-3B

WEHI-3B ($D^-$) is a mineral oil-induced murine myelomonocytic leukemia cell line derived from BALB/c mice. It was originally acquired from Dr. D. Metcalf (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia) and subsequently subcloned at the John Curtin School of Medical Research, Australian National University, Canberra, Australia. One of the subclones designed as WEHI-3B JCS (Leung *et al.*, 1994) was used in this study.

### 2) M1

M1 is a murine myeloblastic leukemia cell line established from a spontaneous myeloid leukemia of SL strain mice (Ichikawa, 1969). It was purchased from the American Type Culture Collection (ATCC), U.S.A.

### 3) HL-60

HL-60 is a human promyelocytic leukemia cell line derived by leukopheresis from a 36-year-old Caucasian female with acute promyelocytic leukemia (Collins *et al.*, 1977). It was purchased from the ATCC, U.S.A.

### 4) NB4

NB4 is a human promyelocytic leukemia cell line established from the bone marrow of a 23-year-old woman with acute promyelocytic leukemia (Lanotte *et al.*, 1991). NB4 cells carry the t(15;17) PML-RARA fusion gene. It was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. The subclone used in this study was obtained from Prof. K.P. Fung (Department of Biochemistry, CUHK).

### 5) K-562

K-562 is a human chronic myelogenous leukemia (CML) cell line established from the pleural effusion of a 53-year-old female with CML in terminal blast crises (Lozzio and Lozzio, 1975). It was purchased from the ATCC, U.S.A.

### 6) U-937

U-937 is a human histiocytic monoblast-like lymphoma cell line originated from the pleural effusion of a patient with histiocytic lymphoma (Sundstrom and Nilsson, 1976). It was purchased from the ATCC, U.S.A.

### 2.1.3 Cell Culture Medium, Buffers and Other Reagents

#### 1) Cell Culture Medium

The powdered form of RPMI 1640 medium (GIBCO BRL Life Technologies Inc.), supplemented with 25 mM N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES) and 2 mM L-glutamine was used for the preparation of culture medium. The powdered medium dissolved in deionized water was buffered with 2 g sodium bicarbonate ($NaHCO_3$) (Sigma Chemical Co.) per litre. The solution was then adjusted to pH 7.14 by 1 M hydrochloric acid (HCl) or sodium hydroxide (NaOH) (Sigma Chemical Co.). After that, it was sterilized by membrane filtration through a 0.22 μm Millipore filter and stored at 4°C until use.

RPMI medium supplemented with 1% antibiotics, which was called "Plain Medium" (PM), was usually used in washing cells. Complete RPMI medium (CM) for cell culture was prepared by supplementing PM with 10% or 20% fetal bovine serum (FBS). Most of the human and murine cell lines were cultured with 10% FBS-supplemented CM while HL-60 was cultured with 20% FBS-supplemented CM. Culture medium used for doing assays was prepared by supplementing PM with 10% or 20% heat-inactivated fetal bovine serum (HI-FBS).

#### 2) Serum Supplements

Fetal Bovine Serum (FBS) (GIBCO BRL Life Technologies Inc.) was stored as 50 ml and 20 ml aliquots. HI-FBS was prepared by heating the FBS at 56°C for 30 minutes. Both of them were stored up at -20°C until use.

### 3) Antibiotic Solutions

Two types of antibiotic solutions were used in alternative periods: antibiotic PSF [10,000 units/ml penicillin G (sodium salt), 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B as Fungizone in 0.85% saline] and antibiotic PSN [5,000 μg/ml penicillin G sodium, 5,000 μg/ml streptomycin sulfate and 10,000 μg/ml neomycin sulfate in 0.85% saline]. The 100X stock solutions were purchased from GIBCO BRL Life Technologies Inc. They were kept at -20°C as 5 ml aliquots.

### 4) Dulbecco's Phosphate-Buffered Saline (PBS)

PBS (Sigma Chemical Co.) was prepared by dissolving the powdered salt (8 g sodium chloride, 0.2 g potassium chloride, 0.2 g monobasic potassium phosphate and 1.15 g dibasic sodium phosphate) in one litre of deionized water. The pH of the solution was adjusted to 7.4 by adding 1 M HCl or NaOH before use. It was sterilized by autoclaving at 121°C for 20 minutes.

### 5) Thioglycollate (TG) Broth

TG broth (3% w/v) was prepared by suspending 3 g of dehydrated thioglycollate powder (Difco Lab.) in 100 ml deionized water. It was then heated to boiling to completely dissolve the powder and sterilized by autoclaving at 121°C for 20 minutes. It was kept in dark at room temperature for at least one month before use.

### 6) Methylthiazoletetrazolium (MTT)

MTT was purchased from Sigma Chemical Co. in powdered form. It was dissolved in deionized water to prepare the stock solution at 5 mg/ml concentration. The stock was stored at 4°C until use.

### 7) Dye Solutions

#### a) Hemacolor Staining Solutions

Three distinct Hemacolor staining solutions (Diagnostica Merck) were used to stain cells after cytocentrifugation. Hemacolor Solution 1 was methanol for fixing cells, Hemacolor Solution 2 was a buffered color reagent red and Hemacolor Solution 3 was a buffered color reagent blue. The buffer solutions were freshly prepared by dissolving 1 buffer tablet in 1 litre of deionized water. They were stable for at least 4 weeks and stored in a tightly closed glass bottle. All staining solutions were light-protected and kept at room temperature.

#### b) Trypan Blue Solution

Trypan blue solution was purchased from Gibco BRL Life Technologies Inc. It contained 0.4% (w/v) trypan blue dissolved in 0.85% saline.

### 8) Linoleic Acid and Conjugated Linoleic Acid

Linoleic acid (LA), a mixture of conjugated linoleic acid isomers (CLA-mix) and a variety of individual CLA isomers employed in this study are shown in Table 2.1. LA and CLA-mix were purchased from Sigma Chemical Co. while CLA isomers, including *cis*-9, *trans*-11 conjugated linoleic acid (9Z, 11E-CLA), *trans*-10, *cis*-12 conjugated linoleic acid (10E, 12Z-CLA), *cis*-9, *cis*-11 conjugated linoleic acid (9Z, 11Z-CLA) and *trans*-9, *trans*-11 conjugated linoleic acid (9E, 11E-CLA), were procured from Matreya, Inc. The composition of the CLA-mix is shown in Table 2.2. All fatty acids were supplied in liquid form and they were used for subsequent cell culture assays by dissolving in absolute ethanol (EtOH). All stock solutions were kept in dark at -20°C.

**Table 2.1: Linoleic acid (LA), a mixture of conjugated linoleic acid isomers (CLA-mix) and the four individual CLA isomers employed in this study.**

| Fatty acids | Estimated purity (%) | Stock Concentration (M) |
|---|---|---|
| LA | 99 | 0.2 |
| CLA-mix | > 99 | 0.2 |
| *cis*-9, *trans*-11 CLA (9Z, 11E-CLA) | > 98 | 0.2 |
| *trans*-10, *cis*-12 CLA (10E, 12Z-CLA) | > 98 | 0.2 |
| *cis*-9, *cis*-11 CLA (9Z, 11Z-CLA) | 98 | 0.2 |
| *trans*-9, *trans*-11 CLA (9E, 11E-CLA) | > 96 | 0.2 |

**Table 2.2: The composition of conjugated linoleic acid (CLA-mix) employed in this study.**

| Fatty Acid | Relative Proportion (%)* |
|---|---|
| *cis*-9, *trans*-11 and *trans*-9, *cis*-11 CLA (9Z, 11E- and 9E, 11Z-CLA) | 41.2 |
| *trans*-10, *cis*-12 CLA (10E, 12Z-CLA) | 44.1 |
| *cis*-9, *cis*-11 CLA (9Z, 11Z-CLA) | 1.1 |
| *cis*-10, *cis*-12 CLA (10Z, 12Z-CLA) | 9.4 |
| *trans*-9, *trans*-11 and *trans*-10, *trans*-12 CLA (9E, 11E- and 10E, 12E-CLA) | 1.3 |
| LA | < 1 |

*Data obtained from Sigma Chemical Co.

### 2.1.4 Reagents for $^{3}$H-Thymidine Incorporation Assay

#### 1) [Methyl-$^{3}$H] Thymidine ($^{3}$H-TdR)

The $^{3}$H-TdR stock solution (Amersham Life Science Ltd.) with specific activity of 2 Ci/mmol was kept as 500 µl aliquots at 4°C. It was freshly diluted with 10% FBS-supplemented complete RPMI medium (10% CM) to make up the 25 µCi/ml working solution for DNA labeling. Twenty microlitres working solution was added into each well of the 96-well flat-bottomed microtiter plate for performing the proliferation assay.

**2) Liquid Scintillation Cocktail**

The ready-to-use optiPhase "High" safe-2 liquid scintillation cocktail, obtained from Perkin-Elmer Co., is based on biodegradable solvents with dioctyl sulfosuccinate, sodium salts and poly(ethyleneglycol)mono(4-nonylphenyl)-ether. It was stored in dark at room temperature.

### 2.1.5 Reagents and Buffers for Flow Cytometry

**1) Propidium Iodide (PI) DNA Staining Buffer**

The PI DNA staining buffer, freshly prepared in PBS, comprised 400 μg/ml ribonuclease A (RNase A) (Boehringer Mannheim), 50 μg/ml propidium iodide (Boehringer Mannheim), 10 mM EDTA (pH 7.4) (Sigma Chemical Co.), 0.1% trisodium citric acid (Sigma Chemical Co.) and 0.1% Triton X-100 (Sigma Chemical Co.). All these reagents were stored up at 4°C until use.

**2) JC-1 Staining Solution**

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) is a cationic dye that exhibits potential-dependent accumulation in mitochondria, as illustrated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). It was purchased from Molecular Probes Inc. (U.S.A). It was dissolved as stock solution at a concentration of 5 mg/ml in DMSO and was kept at -20°C. The dye was diluted to 0.13% as a working solution in warm PBS.

### 3) Fluorescein Diacetate

Fluorescein diacetate, purchased from Calbiochem®, was used for measuring the activity of monocytic serine esterase (also known as non-specific esterase). A stock solution of 20 mg/ml was prepared by dissolving the powder in sterilized DMSO and stored at 4°C.

### 4) Fluorescein Isothiocyanate (FITC)-Conjugated Bovine Serum Albumin (BSA)

FITC-BSA was purchased from Sigma Chemical Co. in powdered form and stored in dark at 4°C. The FITC content was 11.2 mol/mol albumin. It was freshly prepared to a working solution of 1 mg/ml by dissolving in RPMI + 10% HI-FBS when use.

### 5) Dihydroethidium (DHE)

DHE, also known as hydroethidine, was purchased from Molecular Probes Inc. The 10 mM DHE stock solution was prepared by dissolving 1 mg DHE powder in DMSO and was kept at -20 °C in dark. The working concentration of DHE was 10 μM.

### 6) Antibodies

#### a) Mouse IgG

The mouse IgG was purchased from Sigma Chemical Co. It was used to block the non-specific binding of immunoglobulins to Fc receptors of cells. It was stored at -20°C in 0.5 ml aliquots.

**b) Rat IgG**

The rat IgG was purchased from Sigma Chemical Co. It was used to block the non-specific binding of immunoglobulins to Fc receptors of cells. It was stored at -20°C in 0.5 ml aliquots.

**c) Rat Anti-mouse Macrophage Differentiation Antigen CD11b (Mac-1) Monoclonal Antibody (clone M1/70, rat $IgG_{2b}$ isotype)**

The Mac-1 monoclonal antibody was derived from the cell culture supernatant of the hybridoma cell line M1/70, which was purchased from the ATCC, U.S.A. Small volume aliquots of the Mac-1 antibody were kept at -70°C.

**d) Rat Anti-mouse Macrophage Differentiation Antigen F4/80 Monoclonal Antibody (clone C1.A3-1, rat $IgG_{2b}$ isotype)**

The F4/80 monoclonal antibody was derived from the cell culture supernatant of the hybridoma cell line F4/80, which was purchased from the ATCC, U.S.A. Small volume aliquots of the F4/80 antibody were stored at -70°C.

**e) Rat Anti-mouse Myeloid Differentiation Antigen Gr-1 Monoclonal Antibody (clone RB6-8C5, rat $IgG_{2b}$ isotype)**

The Gr-1 monoclonal antibody was purchased from BD Pharmingen. The RB6-8C5 antibody reacts with the myeloid differentiation antigen, Gr-1. It was stored at -70°C until use.

### f) FITC-Conjugated Goat F(ab')$_2$ Anti-Rat IgG Antibody

It was purchased from Southern Biotechnology Associates Inc. and established from the pepsin digest of goat anti-rat IgG (H+L) which was purified by gel filtration. The FITC conjugate was supplied as 0.5 mg in 1 ml PBS at pH 7.4 with 0.1% sodium azide as preservative. It reacts with the heavy and light chains of rat IgG and with the light chains of rat IgM and IgA. It has minimal cross reactivity with mouse immunoglobulins. It was stored at 4°C in dark.

## 7) Antioxidants

### a) Superoxide Dismutase (SOD)

SOD, purchased from Sigma Chemical Co., catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. SOD has been reported to suppress apoptosis in cultured rat ovarian follicles, neural cell lines and transgenic mice (Tilly *et al.*, 1995; Keller *et al.*, 1998). The 200 units/ml SOD working solution was prepared by dissolving 30,000 units/ml SOD powder in 150 ml RPMI medium supplemented with 10% HI-FBS. It was kept at -20°C until use.

### b) N-acetylcysteine (NAC)

NAC, purchased from Sigma Chemical Co., has been reported to increase the cellular pools of free radical scavengers and to prevent apoptosis in neuronal cells (Ferrari *et al.*, 1995). The 15 mM NAC working solution was prepared by dissolving 0.1 g NAC powder in 40.85 ml RPMI + 10% HI-FBS. It was kept at 4°C until use.

### 8) 1% Paraformaldehyde

Paraformaldehyde was purchased from Sigma Chemical Co. in powdered form. One gram paraformaldehyde was dissolved in 50 ml of deionized water by heating to 60°C with stirring in a fume hood for 1 hour. A few drops of 1 M NaOH were added to help to dissolve until a clear solution was obtained. After cooling to room temperature, 50 ml 2X PBS solution was added to produce 1% paraformaldehyde in 1 X PBS and the solution was stored in dark at 4°C until use.

### 9) FACS Medium

The FACS medium contained 2% HI-FBS and 0.05% sodium azide in PBS. The azide was added to inhibit the process of internalization of cell-bound antibodies, as well as patching and capping of the cells. The FACS medium was used for washing and resuspension of cells in FACS analysis and was kept at 4°C.

### 10) FACS Flow Shealth Fluid

The shealth fluid is a ready-to-use solution purchased from Becton Dickinson International. It is a balanced electrolyte solution comprising sodium chloride, potassium chloride, disodium EDTA, sodium fluoride and an anti-microbial agent. The solution was kept at room temperature.

## 2.1.6 Reagents for DNA Extraction

### 1) IGEPAL CA-630 Lysis Buffer

This lysis buffer was purchased from Sigma Chemical Co and "IGEPAL" is a registered trademark of Phone-Poulenc, Inc. The buffer was prepared in 50 mM Tris [hydroxylmethyl] amino methane (Tris)-HCl, pH 7.5 with 3% non-ionic detergent

IGEPAL CA-630 ((Octylphenoxy) polyethoxyethanol) and 20 mM EDTA. It was kept at room temperature.

**2) Proteinase K**

Proteinase K, which is a highly active subtilisin type of protease, was purchased from Boehringer Mannheim in powdered form. It was purified from the mold *Tritirachium album* Limber. The stock solution was prepared at a concentration of 20 mg/ml by dissolving it in autoclaved deionized water and stored as 500 µl aliquots at -20°C until use.

**3) RNase A**

RNase A is a pancreatic RNase powder purchased from Boehringer Mannheim. The stock was prepared at a concentration of 10 mg/ml by dissolving in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl. It was stored as 500 µl aliquots at -20°C until use.

**4) Sodium Acetate Solution (NaOAc)**

The NaOAc acetate solution was purchased from Sigma Chemical Co in powdered form. A 3 M stock solution was prepared by dissolving 24.61 g sodium acetate in 100 ml deionized water. It was then sterilized by autoclaving at 121°C for 20 minutes. The solution was kept at room temperature.

**5) $T_{10}E_{0.1}$ Buffer**

The $T_{10}E_{0.1}$ buffer was made up of 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in deionized water. The solution was kept at room temperature.

### 2.1.7 Cell Death Detection ELISA$^{PLUS}$ Kit

This ELISA kit was purchased from Roche Applied Science, and the whole set was stored at 4°C. The kit contained the following reagents:

**1) Anti-histone-biotin**

This monoclonal antibody, raised from mouse (clone H11-4), was biotin-labeled for the binding of histone component of the nucleosomes and the capturing of immune complex via biotin to the streptavidin-coated microplate. It was reconstituted in 450 µl deionized water when use.

**2) Anti-DNA-POD**

This monoclonal antibody, raised from mouse (clone MCA-33), was conjugated with peroxidase for binding the DNA components of the nucleosomes and for the color reaction with ABTS substrate. It was reconstituted in 450 µl deionized water when use.

**3) Positive Control**

DNA-Histone-Complex, the positive control, reacts positively with the ABTS substrate. It was reconstituted in 450 µl deionized water when use.

**4) Incubation Buffer**

A 100 ml ready-to-use incubation buffer was used to prepare the immunoreagent. The immunoreagent was freshly prepared by mixing 1/20 volume anti-histone-biotin and 1/20 volume anti-DNA-POD with 18/20 volume incubation buffer.

**5) Lysis Buffer**

A 100 ml ready-to-use lysis buffer was used to lyse cells.

**6) Substrate Buffer**

A 15 ml ready-to-use substrate buffer was used to dissolve the ABTS tablets.

**7) ABTS Substrate Tablet**

2, 2'-Azino-bis[3-ethyl-benzothiazoline-6-sulfonate] (ABTS) tablets were dissolved in substrate buffer to give a pale green solution. The substrate solution turns dark green following reaction with peroxidase. The intensity of green color, therefore, could be used to illustrate the degree of apoptosis in this assay. Each tablet was dissolved in 5 ml substrate buffer.

### 2.1.8 Reagents for Measuring Caspase Activity

**1) Cell Lysis Buffer**

This cell lysis buffer consisted of 1% (v/v) IGEPAL-CA 630 (Sigma Chemical Co.), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and one tablet of complete protease inhibitor cocktail, which yielded a mixture of several protease inhibitors with a broad spectrum of activity on serine, cysteine and metalloproteases and calpains inhibitors when dissolved in 50 ml deionized water. The tablet was purchased from Roche Molecular Biochemicals. The cell lysis buffer was stored up at 4°C until use.

**2) Reaction Buffer**

The reaction buffer was made up of 10 mM HEPES-KOH (pH 7.0), 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.1% CHAPS, 100

μg/ml BSA, 10 mM DTT. The solution was kept at 4°C.

**3) Dithiothreitol (DTT)**

DTT was purchased from Invitrogen Life Technologies Inc. It was dissolved in deionized water as 1 M stock was stored up at -20°C. It was added to the reaction buffer to a final concentration of 10 mM just before use for the full activity of the enzymes by stabilizing enzymes with free sulfhydryl groups.

**4) Caspase-3 Substrate, Ac-DEVD-AMC**

Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), purchased from Sigma Chemical Co., is a specific fluorogenic caspase-3 substrate. It was dissolved in DMSO as a 2 mM stock solution and was stored at -20°C.

**5) Caspase-3 Inhibitor, Ac-DEVD-CHO**

Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO), purchased from ALEXIS Biochemicals, is a specific caspase-3 inhibitor. It was dissolved in DMSO as a 10 mM stock solution and was stored at -20°C.

**6) Caspase-8 Substrate, Ac-IETD-AMC**

Ac-Ile-Glu-Thr-Asp-7-Amido-4-methylcoumarin (Ac-IETD-AMC), purchased from ALEXIS Biochemicals, is a fluorogenic substrate for caspase-8 and granzyme B. It was dissolved in DMSO as a 1 mM stock solution and was stored at -20°C.

**7) Caspase-8 Inhibitor, Ac-IETD-CHO**

Ac-Ile-Glu-Thr-Asp-CHO, purchased from ALEXIS Biochemicals, is a

caspase-8 and granzyme B inhibitor. It was dissolved in DMSO as a 10 mM stock solution and was stored at -20°C.

**8) Caspase-9 Substrate, Ac-LEHD-AFC**

Ac-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (Ac-LEHD-AFC), purchased from ALEXIS Biochemicals, is a fluorogenic substrate for caspase-9. It was dissolved in DMSO as a 5 mM stock solution and was stored at -20°C.

**9) Caspase-9 Inhibitor, Ac-LEHD-CHO**

Ac-Leu-Glu-His-Asp-CHO, purchased from ALEXIS Biochemicals, is a caspase-9 inhibitor. It was dissolved in DMSO as a 10 mM stock solution and was stored at -20°C.

**10) 7-Amino-4-Methyl Coumarin (AMC)**

AMC is a standard marker for quantifying the activities of caspase-3 and caspase-8. It was dissolved in DMSO as a 0.05 M stock solution and was further diluted with lysis buffer to 100 μM. Both the stock and the diluted solutions were kept at 4°C.

**11) 7-Amino-4-trifluoromethyl Coumarin (AFC)**

AFC is a yellow powder that is used as a standard marker for quantifying the activity of caspase-9. It was dissolved in methanol as a 0.02 M stock solution and was further diluted with lysis buffer to 80 μM. Both the stock and the diluted solutions were kept at 4°C.

### 2.1.9 Reagents for Total RNA Isolation

#### 1) DEPC-treated Deionized Water

Deionized water was treated with 0.1% diethyl pyrocarbonate (DEPC) which was purchased from Sigma Chemical Co. After shaking thoroughly to disperse the DEPC, it was allowed to stand overnight. The solution was then autoclaved at 121°C for 20 minutes in order to destroy the remaining DEPC.

#### 2) Trizol Reagent

Trizol reagent, purchased from Invitrogen Life Technologies Inc., is a mono-phasic solution of phenol and guanidine isothiocyanate. This ready-to-use solution maintains the integrity of the RNA, while disrupting cells and dissolving cell components. It was stored in dark at 4°C until use.

#### 3) Chloroform

Chloroform, purchased from BDH Laboratory Supplies with 99.0 to 99.4% purity, was used along with the Trizol reagent to isolate RNA.

#### 4) Isopropanol

Isopropanol, purchased from BDH Laboratory Supplies with 99.7% purity, was used to precipitate RNA.

### 2.1.10 Reagents and Buffers for RT-PCR

#### 1) Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)

The enzyme was purchased from Invitrogen Life Technologies Inc. It was isolated from *Escherichia coli* expressing a portion of the *pol* gene of M-MLV on a

plasmid. This enzyme was stored in a buffer of 20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.01% (v/v) NP-40, 0.1 mM disodium EDTA, 0.1 M sodium chloride and 50% (v/v) glycerol at -20°C. One unit of its activity was defined as the amount of enzyme that would incorporate 1 mole of deoxythymidine triphosphate (dTTP) into acid-precipitable material in 10 minutes at 37°C using poly(A) and oligo-$dT_{12-18}$ as template and primer respectively.

**2) First Strand Buffer (5X)**

The first strand buffer was purchased from Invitrogen Life Technologies Inc. and was supplied as a 5X solution of 250 mM Tris-HCl (pH 8.3), 375 mM potassium chloride and 15 mM magnesium chloride ($MgCl_2$). It was stored at -20°C until use.

**3) Oligo-$dT_{12-18}$, Sodium Salt ($pd(T)_{12-18}$, sodium salt)**

It was purchased from Promega Corporation and was prepared as a stock solution of 1 μg/μl in $T_{10}E_{0.1}$ buffer. It was stored at -20°C until use.

**4) $T_{10}E_1$ Buffer**

The $T_{10}E_1$ buffer was made up of 10 mM Tris-HCl (pH 8) and 1 mM EDTA in DEPC-treated deionized water. All the constituent chemicals were purchased from Sigma Chemical Co.

**5) $RN_{ASE}OUT^{TM}$ Recombinant Ribonuclease Inhibitor**

It was purchased from Invitrogen Life Technologies Inc. It was affinity purified from a recombinant strain of *E. coli* expressing a cloned porcine liver gene. This inhibitor has a very high binding affinity for pancreatic-type ribonucleases such as

RNase A and is active against RNase A, RNase B and RNase C. It had a concentration of 40 units/μl but a minimum of 1 mM DTT was required to maintain its activity. It was stored at -20°C in a buffer containing 20 mM Tris-HCl (pH 8), 50 mM KCl, 0.5 mM EDTA, 8 mM DTT and 50% (v/v) glycerol. One unit was defined as the amount of inhibitor required to inhibit by 50% the activity of 5 ng of RNase A as determined by the inhibition of hydrolysis of cytidine 2', 3'-cyclic monophosphate by RNase A.

### 6) Thermoprime $^{plus}$ DNA Polymerase

The enzyme, purchased from Advanced Biotechnologies Ltd, is a thermostable DNA polymerase isolated from thermophilic bacteria. It is a single polypeptide of approximately 94 kDa which has 5' to 3' polymerization-dependent exonuclease replacement activity, but lacks the 3' to 5' exonuclease activity. One unit of the enzyme was defined as the amount that would incorporate 10 nmoles of dNTPs into acid insoluble material in 30 minutes at 47°C. It was stored at -20°C until use.

### 7) Reaction Buffer IV (10X)

The reaction buffer, purchased from Advanced Biotechnologies Ltd., was supplied as a 10X solution of 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl (pH 9) and 0.1% (w/v) Tween. It was stored at -20°C until use.

### 8) $MgCl_2$ solution

It was purchased from Advanced Biotechnologies Ltd. at 25 mM concentration and was stored at –20°C until use.

**9) Ultrapure dNTP Set, 2'-Deoxyribonucleoside 5'-Triphosphate, Sodium Salt**

It was purchased from Pharmacia Biotech, with each nucleotide supplied as 100 mM solution in deionized water (pH 7.5). Ten microlitres of each nucleotide stock solution was mixed with 60 μl $T_{10}E_1$ buffer to make a working solution. The working solution was stored at -20°C.

**10) Primer Pairs**

The specific pairs of oligonucleotide primers were designed on the basis of the published sequences of cloned cDNA. They were devised to prime on the sense and antisense sequences of the corresponding cDNA respectively. The PCR primer sets for PCR amplifications of specific cDNA were synthesized by Invitrogen Life Technologies Inc. The lyophilized primer pairs were reconstituted in DEPC-treated deionized water to a working concentration of 2.5 μM. The primers were stored as 500 μl aliquots at -20°C. They have the annealing temperature of 56-61°C. The sequences of the primer pairs and the predicted sizes of the PCR products are summarized in Table 2.3.

**Table 2.3: Primers used in RT-PCR and the predicted sizes of the PCR products.**

| cDNA amplified | Sequence (5' to 3') | Predicted size of the amplified PCR product (bp) |
|---|---|---|
| GAPDH | Sense strand:<br>AAT GGT GAA GGT CGG TGT GAA C<br>Antisense strand:<br>GAA GAT GGT GAT GGG CTT CC | 226 |
| p21 | Sense strand:<br>CTT TGA CTT CGT CAC GGA GAC<br>Antisense strand:<br>AGG CAG CGT ATA TCA GGA GAC | 469 |
| p27 | Sense strand:<br>AAG CAC TGC CGG GAT ATG GA<br>Antisense strand:<br>AAC CCA GCC TGA TTG TCT GAC | 293 |
| Bcl-xL | Sense strand:<br>TGG CAA CCC ATC CTG GCA CCT<br>Antisense strand:<br>ACT GAA GAG TGA GCC CAG CAG AAC | 538 |
| Bak | Sense strand:<br>GCC CAG GAC ACA GAG GAG GTT TTC<br>Antisense strand:<br>AAA CTG GCC CAA CAG AAC CAC ACC | 528 |
| Bad | Sense strand:<br>ATC CCA GAG TTT GAG TCG AGG GA<br>Antisense strand:<br>TTC GAG CCC ACC AGG ACT GGA | 445 |
| TNF-α | Sense strand:<br>TCC CCA AAG GGA TGA GAA GTT C<br>Antisense strand:<br>TCA TAC CAG GGT TTG AGC TCA G | 411 |
| IL-1β | Sense strand:<br>GAG CTT CAG GCA GGC AGT ATC<br>Antisense strand:<br>GTA TAG ATT CTT TCC TTT GAG GC | 582 |
| IFN-γ | Sense strand:<br>AGGAACTGGCAAAAGGATGGTG<br>Antisense strand:<br>GTGCTGGCAGAATTATTCTTATTG | 353 |

### 2.1.11 Reagents and Buffers for Gel Electrophoresis of Nucleic Acids

#### 1) Tris-Borate-EDTA (TBE) Electrophoresis Buffer (5X)

The buffer stock was 5X which was prepared by dissolving 54 g Tris, 27.5 g boric acid and 20 ml 0.5 M EDTA in 1 litre deionized water. The pH of the buffer was adjusted to 8. The concentration of the working TBE buffer was 0.5X and it was prepared by diluting one part of the 5X stock TBE buffer with nine parts of deionized water. Both of the working and stock solutions were kept at 4°C.

#### 2) Gel Loading Dye (5X)

The gel loading dye, purchased from Sigma Chemical Co., was a 5X solution containing 0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 M EDTA (pH 8.0) and 0.5% sodium dodecyl sulfate (SDS). The solution was used for non-denaturing agarose gel electrophoresis of nucleic acids.

#### 3) 100 bp DNA Marker

The 100 bp DNA marker was purchased from Invitrogen Life Technologies Inc. The stock concentration was 1 μg/μl, and the working solution was prepared by diluting the stock solution 10 fold with $T_{10}E_1$ buffer.

#### 4) Agarose Gel

The agarose powder was purchased from Sigma Chemical Co. The 1% (w/v) and 2% (w/v) agarose gels were prepared by dissolving 10 g and 20 g agarose in 1 litre 0.5X TBE buffer respectively, which were then heated to 70°C on a hot plate with constant stirring until the solution became clear. The 1% agarose gel was used for RNA gel electrophoresis while the 2% agarose gel was used for DNA and PCR

products gel electrophoresis.

**5) Ethidium Bromide (EtBr)**

It was purchased from Sigma Chemical Co. The stock concentration was 10 mg/ml and was prepared by dissolving the EtBr in deionized water. The working solution was prepared through dilution of the stock solution by 10,000 fold. Both the stock and working solutions were kept in dark at room temperature.

### 2.1.12 Reagents, Buffers and Materials for Western Blot Analysis

**1) Cell Lysis Buffer**

The cell lysis buffer was made up of 1% (v/v) IGEPAL-CA 630 (Sigma Chemical Co.), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and one tablet of complete protease inhibitor cocktail (Roche Molecular Biochemicals) in 50 ml deionized water. Following dissolution in 50 ml lysis buffer, each tablet of complete protease inhibitor cocktail yielded a mixture of several protease inhibitors with a broad spectrum of activity on serine, cysteine and metalloproteases and calpains inhibitors. The cell lysis buffer was kept at 4°C until use.

**2) Bradford Solution**

The Bradford solution, purchased from Bio-Rad Laboratories, is a 450 ml ready-to-use solution. It was stored at 4°C until use.

**3) 30% (w/v) Acrylamide / Bis Solution, 37.5:1**

The acrylamide solution, purchased from Bio-Rad Laboratories, is a 500 ml ready-to-use solution containing 30% (w/v) of acrylamide (146.1 g) and

N,N'-methylene-bis-acrylamide (3.9 g) for a total monomer to crosslinker ratio of 37.5:1. It was kept in dark at 4°C until use.

**4) Lower Buffer for Separating Gel**

The lower buffer, purchased from Sigma Chemical Co., is a 1.5 M Tris-HCl buffer with pH adjusted to 8.8. It was kept at 4°C until use.

**5) 10% Sodium Dodecyl Sulfate (SDS) Solution**

SDS was purchased from Sigma Chemical Co. in powdered form. A 10% SDS solution (w/v) was prepared by dissolving 10 g SDS in 100 ml deionized water. It was kept at room temperature.

**6) 10% Ammonium Persulfate (APS)**

Ammonium persulfate was purchased from Bio-Rad Laboratories. The 10% APS solution (w/v) was prepared by dissolving 0.5 g APS in 5 ml deionized water. The solution was kept in 500 μl aliquots at -20°C until use.

**7) N,N,N',N'-Tetra-methylethylenediamine (TEMED)**

It was purchased from Bio-Rad Laboratories and was stored at 4°C until use.

**8) Upper Buffer for Stacking Gel**

The upper buffer, purchased from Sigma Chemical Co., is a 0.5 M Tris-HCl buffer with pH adjusted to 6.8. It was stored up at 4°C.

**9) 4X SDS Sample Loading Buffer**

It was prepared by mixing 0.4 ml 0.05% (w/v) bromophenol blue, 2 ml upper buffer, 2 ml glycerol, 2 ml 10% (w/v) SDS, 1.4 ml deionized water and 0.2 ml 2-mercaptoethanol. All reagents were purchased from Sigma Chemical Co. The loading buffer was stored at 4°C until use.

**10) Kaleidoscope Prestained Standards**

These prestained standards, purchased from Bio-Rad Laboratories, consisted of seven uniquely colored proteins including myosin (199,000 Da), β-galactosidase (128,000 Da), bovine serum albumin (85,000 Da), carbonic anhydrase (41,700 Da), soybean trypsin inhibitor (32,100 Da), lysozyme (18,300 Da) and aprotinin (7,500 Da). The standard colored proteins prepared in 33% (v/v) glycerol, 3% SDS, 10 mM Tris (pH 7), 10 mM DTT, 2 mM EDTA, 0.01% $NaN_3$ were stored at -20°C until use.

**11) Tris-Glycine-SDS Electrophoresis Buffer (10X)**

The 10X buffer contained 0.25 M Tris-HCl, pH 8.6, 1.92 M glycine and 1% SDS in deionized water. It was filtered by filtration through a 0.2 μm Millipore filter and was stored at 4°C. The 10X concentrate was freshly diluted to 1X working buffer solution for SDS-PAGE.

**12) Coomassie Blue Staining and Destaining Solutions**

The Coomassie blue staining and destaining solutions were used to stain and destain the SDS-polyacrylamide gel respectively after transferring the proteins from the gel to the PVDF membrane. The staining solution was prepared by mixing one part of 0.05% Coomassie blue (Bio-Rad Lab.) in acetic acid, three parts of methanol

and ten parts of deionized water. For the destaining solution, it was prepared by mixing one part of acetic acid, three parts of methanol and ten parts of deionized water.

**13) Tris-Glycine Buffer (10X)**

The Tris-glycine buffer was prepared by mixing 0.25 M Tris-HCl (pH 7.5) with 1.92 M glycine in deionized water. It was then filtered by filtration through a 0.22 μm Millipore filter and stored up at 4°C.

**14) Tris-Glycine-Methanol Transfer Buffer (1X)**

The Tris-glycine-methanol transfer buffer was prepared by mixing 100 ml methanol with 50 ml 10X Tris-glycine buffer in 350 ml deionized water. The buffer solution was stored at 4°C.

**15) Polyvinylidene Difluoride (PVDF) Western Blotting Membranes**

The microporous PVDF membrane with pore size of 0.45 μm was purchased from Roche Applied Science and was stored at room temperature.

**16) TBS-Tween Washing Buffer (10X)**

The washing buffer was made up of 100 mM Tris-HCl (pH 7.5), 1 M NaCl and 1% (v/v) Tween 20 in deionized water. All the constituent reagents were purchased from Sigma Chemical Co. The working solution (1X) was prepared through dilution of the 10X stock by 10 fold using deionized water. Both the stock and working solution were stored up at 4°C.

**17) 5% Skimmed Milk Solution (Blocking Solution)**

It was prepared by dissolving Nestle's skimmed milk powder in 1X washing buffer at a concentration of 5% (w/v).

**18) Primary Antibodies**

**a) Rabbit Anti-FasL Polyclonal Antibody**

This anti-FasL antibody, purchased from Santa Cruz Biotechnology, Inc., is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 100-278 mapping at the carboxyl terminus of FasL of rat origin. Each vial contained 200 μg IgG in 1 ml PBS, 0.1% sodium azide and 0.2% gelatin. It reacts with FasL of mouse, rat and human origin. The antibody was stored at 4°C.

**b) Rabbit Anti-Fas Polyclonal Antibody**

This anti-Fas antibody, purchased from Santa Cruz Biotechnology Inc., is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1-335 representing full length Fas of human origin. Each vial contained 200 μg IgG in 1 ml of PBS, 0.1% sodium azide and 0.2% gelatin. The antibody reacts with Fas of human, mouse and rat origin. The antibody was stored at 4°C.

**c) Mouse Anti-β actin Monoclonal Antibody**

This anti-β-actin antibody, purchased from Sigma Chemical Co., is a monoclonal antibody (mouse $IgG_{2a}$ isotype) derived from the AC-74 hydridoma as a result of fusion of mouse myeloma cells and splenocytes from an immunized mouse. It recognizes an epitope located on the N-terminal end of the β-isoform of actin. The antibody specifically reacts with β-actin (42 kDa) in a wide variety of tissues and

species using immunoblotting. The product was provided as ascites fluid containing 0.1% sodium azide as a preservative.

### 19) Secondary Antibodies

Two types of secondary antibodies were used in this study. These include sheep anti-mouse IgG, peroxidase-linked species-specific whole antibody and donkey anti-rabbit IgG, peroxidase-linked species-specific whole antibody. Both of them were purchased from Amersham Pharmacia Biotech. with the concentration of 1 mg/ml. They were used in 1:1,000 concentration. The antibodies were stored up at 4°C.

### 20) Western Blotting Luminol Reagents

The Western blotting luminol reagents, purchased from Santa Cruz Biotechnology Inc., consisted of solution A (125 ml) and solution B (125 ml). They were kept at 4°C. An equal volume of solution A and B was mixed well before use.

### 21) X-ray Films (Fuji, Japan)

The Fuji X-ray films were stored in dark at 4 °C.

## 2.2 Methods

### 2.2.1 Culture of the Tumor Cell Lines

All the murine and human leukemia cell lines (WEHI-3B JCS, M1, NB4, K-562), and the human lymphoma cell line (U-937) were maintained in complete RPMI medium supplemented with 10% FBS, 1% PSF or PSN as continuous suspension cultures. For the human leukemia HL-60 cells, they were maintained in the same condition except that 20% FBS was mandatory for their normal growth. All cell lines

were cultured in 25 or 75 $cm^2$ tissue culture flasks and incubated at 37°C in a humidified incubator supplied with 5% carbon dioxide ($CO_2$). The cell lines were sub-cultured at 2-3 day intervals or twice weekly depending on their doubling times. Cells in the exponential growth phase were used for all experiments. Long term storage of cell lines was done by cryo-preservation in liquid nitrogen.

### 2.2.2 Isolation, Preparation and Culture of Mouse Peritoneal Macrophages

BALB/c mice were injected intraperitoneally (i.p.) with 1-1.5 ml sterile 3% thioglycollate broth to elicit the peritoneal macrophages. Three days later, the mice were sacrificed by cervical dislocation and placed ventral side up on a clean surface. The skin of the abdomen was cleaned with 70% ethanol, cut and pulled apart so that the abdominal wall was exposed. A 5 ml syringe fitted with a 20-gauge needle was used to inject some air to inflate the peritoneal cavity. After that, 3 ml plain RPMI medium was injected into the abdominal cavity to wash out the cells and the procedure was repeated twice to rinse out most of the peritoneal exudate cells (PEC). The thioglycollate-elicited peritoneal macrophages were then washed twice with cold plain RPMI medium. They were finally resuspended in RPMI medium and supplemented with 10% HI-FBS and seeded into a 96-well plate microtiter at a concentration of $5x10^5$ cells/ml for 3 hours to allow the adherence of macrophages to the bottom of the wells. The non-adherent cells were then removed and different concentrations of CLA-mix were added for further analysis.

### 2.2.3 Determination of Cell Viability

#### 1) MTT Colorimetric Assay

All the murine and human leukemia or lymphoma cell lines ($10^4 - 10^5$ cells/ml) as well as the thioglycollate-elicited murine peritoneal macrophages ($2.5 \times 10^6$ cells/ml) were incubated alone or with different concentrations of CLA-mix and its isomers (0-200 μM) respectively in the 96-well flat-bottomed microtiter plates at 37°C for a defined incubation period (24, 48 or 72 hours) inside a humidified 5% $CO_2$ incubator. The cells were then incubated with 30 μl MTT (5 mg/ml) for 2-3 hours. Then the medium was removed and 100 μl DMSO was used to dissolve the MTT deposited in each well. Then absorbance was measured at 540 nm. The viability of cells was calculated as follows:

% viable cells = ($OD_{540}$ of test sample / $OD_{540}$ of control) × 100%

#### 2) Trypan Blue Exclusion Assay

The trypan blue exclusion test was used to determine the number of viable cells in cultures. Cells were cultured with different concentrations of CLA-mix (0-200 μM) at 37°C inside a humidified 5% $CO_2$ incubator. The viability of the cells was then determined after a defined incubation period (24, 48 or 72 hours). Ten microlitres cell suspension was mixed with 10 μl of 0.4% trypan blue solution. The viable cells were visible as clear cell bodies while the dead cells were stained blue. By counting the number of viable and dead cells, the viability was calculated as follows:

% cell viability = (no. of viable cells / no. of total cells counted) × 100%

### 2.2.4 Determination of Cell Proliferation by [$^3$H]-TdR Incorporation Assay

Leukemia WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix (0-200 μM) in the 96-well flat-bottomed microtiter plates at 37°C for 72 hours inside a humidified 5% $CO_2$ incubator. The cells were then pulsed with 0.5 μCi [$^3$H]-TdR in 20 μl complete medium for 6 hours. After a freeze-and-thaw cycle, the cells were harvested onto a glass microfiber filter. The radioactivity, expressed in counts per minute (cpm), was measured by the liquid scintillation analyzer LS 2900 TR. The results were expressed as the percentage inhibition of [$^3$H]-TdR incorporation, using the untreated cells as a control (Leung *et al.*, 1994). The percentage inhibition of [$^3$H]-TdR incorporation was calculated as follows:

% inhibition = (cpm of control – cpm of test sample) / cpm of control × 100%

### 2.2.5 *In Vivo* Tumorigenicity Study

WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with CLA-mix (100 μM or 150 μM) for 8 hours at 37°C inside a humidified 5% $CO_2$ incubator. The cells were then harvested and washed three times with plain RPMI medium. Each BALB/c mouse in groups of five was injected i.p. with 3 x $10^6$ syngeneic WEHI-3B JCS cells in 1 ml RPMI medium. On day 12 of post-tumor inoculation, WEHI-3B JCS cells were recovered from the peritoneal cavity of mice and the viable tumor cells were counted by trypan blue exclusion assay.

### 2.2.6 Analysis of Cell Cycle Profile / DNA Content by Flow Cytometry

WEHI-3B JCS cells ($10^4$ cells/ml) were first synchronized by culturing in RPMI medium supplemented with 0.5% HI-FBS for 24 hours. They were then incubated in

RPMI + 10% HI-FBS with different concentrations of CLA-mix and its isomers (0-200 μM) for another 24 hours at 37°C inside a humidified 5% $CO_2$ incubator. Cells were harvested and washed with PBS by spinning at 430 × g for 5 minutes. Cells were then fixed with 1 ml 70% ethanol at 4°C for at least 30 minutes. Afterwards, cells were centrifuged at 430 × g for 10 minutes, followed by washing with PBS and centrifuging again at 430 × g for 10 minutes to remove the ethanol. Cells were then resuspended in 1 ml freshly prepared propidium iodide DNA staining solution (50 μg/ml PI) in dark at room temperature for 1.5 hours. Then flow cytometric analysis was carried out. Stained cells were analyzed for fluorescence intensity with a fluorescence-activated cell sorter (Becton Dickinson FACSort) equipped with an argon laser emitting at 488 nm, using the CellQuest software. A minimum of 10,000 events were acquired for each determination. The percentages of cells in $G_0/G_1$, S and $G_2/M$ cell cycle phases were calculated by the ModFit program (Becton Dickinson).

### 2.2.7 Measurement of Apoptosis

#### 1) DNA Fragmentation Analysis

Apoptotic cells show a typical occurrence of fragmented DNA, therefore, DNA fragmentation is regarded as one of the indications of cells undergoing apoptosis. Apoptotic DNA fragments were isolated from the apoptotic cells by the method of Herrmann *et al.* (1994). WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with various concentrations of CLA-mix and its isomers respectively at 37°C for different periods of time and the untreated cells acted as a control. WEHI-3B JCS cells ($3 \times 10^6$) were then harvested and washed with cold PBS by centrifugation at 430 × g for 5 minutes. The cell pellets were then treated with 200 μl IGEPAL CA-630 lysis buffer for 10 minutes at 37°C. The samples were centrifuged at 6,000 × g for 5 minutes.

Supernatants, which contained the apoptotic DNA fragments, were collected to another new eppendorfs and 50 μl 5% SDS was added. The mixture was incubated with 10 μl RNase A (10 mg/ml) at 56°C for 1.5 hour to remove the cellular RNA, followed by another 1.5 hours of incubation with 20 μl proteinase K (20 mg/ml) at 56°C in order to remove the proteins. The DNA was then precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. After centrifugation at 20,800 × g for 30 minutes, the DNA pellets were washed with 70% ethanol and then absolute ethanol, followed by air drying for about 10 minutes. The dried pellets were resuspended in 20 μl $T_{10}E_{0.1}$ buffer and incubated at 37°C for 30 minutes. Just before performing the gel electrophoresis, the samples were heated at 65°C for 5 minutes. After adding 5 μl of gel loading dye into each sample, the DNA samples were loaded into the wells of 2% agarose gel for electrophoresis at 100 volts. After electrophoresis, the gels were stained with ethidium bromide (1 μg/ml) for 3 minutes, followed by destaining with deionized water for 15 minutes. The DNA bands were visualized with UV illumination and the images were captured by Bio-Rad Gel Doc 2000 under UV illumination.

**2) Cell Death Detection ELISA$^{PLUS}$ Kit**

This method can be used to quantify the degree of apoptosis (Bourré *et al.*, 2002). The kit is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies to specifically determine the amount of mono- and oligonucleosomes produced during apoptosis, which are in the cytoplasmic fraction of cell lysates. Briefly, WEHI-3B JCS cells ($10^4$/ml) were either treated with CLA-mix or its isomers (150 μM) for 48 hours, and $10^5$ treated cells were harvested by spinning at 200 × g for 10 minutes. The pellet was resuspended in 200 μl lysis buffer and lysed

for 30 minutes at room temperature. The lysate was then centrifuged at 200 × g for 10 minutes. Twenty microlitres supernatant, which corresponded to the cytoplasmic fraction, was carefully transferred into the streptavidin coated microplate. An equal volume (20 µl) of positive control was also added to a new well while the background control was made by only adding the incubation buffer into the well. After the addition of 80 µl freshly prepared immunoreagent into each well, the plate was covered with the adhesive cover foil provided in the kit and was incubated on a shaker with gentle shaking (300 rpm) for 2 hours at room temperature. Afterwards, the solution was removed thoroughly by aspiration and each well was rinsed 3 times with 250 µl incubation buffer. Then 100 µl ABTS substrate solution was subsequently added into each well. After gentle shaking for 25 minutes, the absorbance was read at 405 nm – 490 nm. The results were converted to enrichment factor which reflected the degree of apoptosis. The enrichment factor was calculated as follows:

$$\text{Enrichment factor} = \frac{\text{Absorbance of sample} - \text{Absorbance of background}}{\text{Absorbance of control} - \text{Absorbance of background}}$$

Enrichment factor greater than or equal to 2 was considered significant when compared with the untreated control (Bourré *et al.,* 2002)

### 2.2.8 Determination of the Mitochondrial Membrane Potential

The mitochondrial membrane potential ($\Delta\psi m$) of CLA-mix- or CLA isomer-treated WEHI-3B JCS cells was measured by the JC-1 staining method (Dorrie *et al.*, 2001). JC-1 stock solution (1.3 µl, 5 mg/ml in DMSO) was diluted with 1 ml warm PBS. WEHI-3B JCS cells ($10^6$) were harvested and centrifuged. The pellet was resuspended with 1 ml working solution and then incubated for 15 minutes at 37°C. After incubation, the cells were subjected to flow cytometric analysis.

Excitation was made at 488 nm and the emissions of red (~590 nm) and green (~525 nm) fluorescence were measured simultaneously.

### 2.2.9 Measurement of Caspase Activity

There were 2 steps in studying the caspase activity:

**1) Treatment of Cells and Extraction of Proteins**

The method for measuring caspase activity was adopted from Mack *et al.* (2000). WEHI-3B JCS cells ($3 \times 10^6$) were treated with CLA-mix or CLA isomer for defined periods of time at 37°C in a humidified $CO_2$ incubator. The cells were harvested and washed twice with cold PBS by centrifuging at 430 × g for 5 minutes at 4°C. Cell viability was determined by the trypan blue exclusion test. The cell pellet was then resuspended in 150 µl lysis buffer (50 µl / $10^6$ cells) and the mixture was vortexed vigorously. The cell lysates were kept on ice for 10 minutes and then centrifuged at 18,000 × g for 3 minutes at 4°C. The supernatants were collected for further use.

**2) Fluorometric Measurement of Caspase Activity**

In order to confirm the correlation between caspase activities with the signal detection, each sample would have its respective control in the presence of specific caspase inhibitor. The control reaction was set up by incubating 50 µl cell lysate with 1 µl of the corresponding caspase inhibitor and 50 µl reaction buffer in a 96-well flat-bottomed microtiter plate at 37°C for 30 minutes. Afterwards, another 50 µl of each cell lysate sample was added into the new wells of the plate. These samples were free from caspase inhibitors. One microlitre of the corresponding specific caspase substrate was then added to all of the wells, and the reaction buffer was added to make up the final volume of 200 µl. The whole mixture was again incubated at 37°C

for 1 hour. The fluorescence units released by the mixture were determined by the fluorescent plate reader, TECAN Polarion. The amount of fluorescence units released represented the caspase activity which could be quantified by comparing with the standard curve of free fluorescence units. The standard curve was made by diluting the AMC or AFC solution to 0.5 μM, 1 μM, 2 μM and 4 μM with the lysis buffer.

### 2.2.10 Study of Intracellular Accumulation of Reactive Oxygen Species (ROS)

The method for measuring the generation of ROS, particularly the superoxide anions ($O_2^-$), was adopted from Ito *et al.* (2004). Dihydroethidium (DHE) enters cells and is oxidized by ROS, with a relative selectivity for $O_2^-$ to form ethidium, which intercalates with DNA and causes the nucleus to exhibit a red shift in fluorescence. DHE and ethidium are retained within cells with minimal leakage. Therefore, to measure the generation of ROS, CLA-mix- or CLA isomer-treated WEHI-3B JCS cells ($5 \times 10^5$) were incubated with 10 μM dihydroethidium (Molecular Probes, Eugene, OR) for 15 minutes at 37°C in a humidified $CO_2$ incubator. The cells were then washed and resuspended in PBS. The oxidative conversion of dihydroethidium to ethidium was analyzed by flow cytometry.

### 2.2.11 Study of the Scavenging Activity of Antioxidants

Prior to treatment with CLA-mix or CLA isomer for 24 hours, WEHI-3B JCS cells ($5 \times 10^5$) were pre-incubated with either 200 units/ml SOD for 3 hours or 15 mM NAC for 2 hours in a humidified $CO_2$ incubator. Afterwards, the cells were cultured with 10 μM dihydroethidium (Molecular Probes, Eugene, OR) for 15 minutes at 37°C, and were then washed and resuspended in PBS. The oxidative conversion of dihydroethidium to ethidium was analyzed by flow cytometry.

### 2.2.12 Gene Expression Study

There were 4 steps in studying gene expression:

**1) Isolation of Total Cellular RNA**

For WEHI-3B JCS cells, $10^6$ cells were firstly treated with appropriate concentrations of CLA-mix or CLA isomer for different periods of time at 37°C inside a humidified 5% $CO_2$ incubator. The cells were then harvested by centrifugation at 430 × g for 5 minutes at 4°C and lysed by adding 1 ml TRIZOL reagent (1 ml TRIZOL for 5-10 x $10^6$ cells) with vigorous shaking. The cell lysate solutions were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Then 200 μl chloroform per 1 ml TRIZOL reagent was subsequently added to the samples and shaked robustly for 15 seconds. The samples were allowed to stand at room temperature for 3 minutes and then centrifuged at 12,000 × g for 15 minutes at 4°C. The upper aqueous phase containing RNA (~400 μl) was being transferred to another new micro-centrifuge tube. Isopropanol (500 μl) was added to precipitate the RNA and the samples were allowed to stand at room temperature for 10 minutes, followed by centrifugation again at 12,000 × g at 4°C for another 10 minutes. The supernatants were then discarded and the RNA pellets were washed with 1 ml 75% ethanol per ml of TRIZOL. After centrifugation at 7,500 × g at 4°C for 5 minutes, the supernatants were removed by aspiration and the RNA pellets were air-dried for about 5 minutes. After that, the RNA pellets were dissolved in 30 μl DEPC-treated water and stored at -70°C until use.

In order to determine the RNA content in each sample, they were firstly diluted 100 fold with DEPC-treated water. The absorbance of the diluted RNA was calculated at 260 nm. According to the measurement results, small aliquots of RNA with a working concentration of 0.5 μg/μl were prepared with DEPC-treated water and kept

at -70°C. The purity was assessed by measuring the ratio of $A_{260}/A_{280}$ (it should have a value ranged from 1.0 to 2.0) and the integrity of RNA was verified by running 1 μl total RNA (0.5 μg) samples on a 1% agarose gel, followed by staining with ethidium bromide.

**2) Reverse Transcription (RT)**

In this step, the RNA was converted to its complementary DNA (cDNA) for further PCR amplification of the target genes. Total RNA (2 μg) were reverse transcribed in a 20 μl reaction mixture containing 40 units of $RN_{ASE}OUT^{TM}$ recombinant ribonuclease inhibitor, 1X M-MLV first strand buffer, 0.5 mM of each dNTP, 10 mM DTT, 0.1 μg $oligo(dT)_{12-18}$ and 200 units of M-MLV reverse transcriptase. A negative control devoid of RNA samples was used to check for contaminations if any. The reaction was carried out in a thermocycler, GeneAmp PCR System 9700 (Perkin-Elmer Co.). Briefly, the reaction mixture was incubated at 37°C for 1 hour, followed by 99°C for 5 minutes to inactivate the reverse transcriptase and to completely denature the template, and then the samples were cooled down to 4°C. The resulting cDNA samples were kept at -20°C until use.

**3) Polymerase Chain Reaction (PCR)**

Two microlitres of each cDNA sample (equivalent to 0.1 μg of total RNA) was mixed with a 23 μl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of $Thermoprime^{Plus}$ DNA polymerase, and 0.2 μM of each sense and antisense oligonucleotide primer in a PCR tube. A negative control exclusive of cDNA sample was prepared to check for any contaminations. The reactions were performed in a thermocycler, GeneAmp PCR System 9700 (Perkin-Elmer Co.).

Briefly, the samples were firstly subjected to an initial denaturation at 94°C for 5 minutes. Then 20-30 thermal cycles were carried out (The number of cycles varied from gene to gene). The thermal cycle basically included denaturation at 94°C for 30 seconds, annealing at 56°C to 61°C for 60 to 75 seconds (the temperature and duration depended on which target genes were amplified) and elongation at 72°C for 1 minute. In the last cycle, one more step in elongation for 5 minutes was performed to complete the reaction. The amplified PCR products were then cooled down to 4°C and kept at 4°C for doing subsequent gel electrophoresis.

**4) Agarose Gel Electrophoresis**

Each PCR product (5 μl) was mixed with 1 μl 5X gel loading buffer. The mixture was then added into the wells of the 2% agarose gel. Simultaneously, a mixture of 5 μl 100 bp DNA ladder and 1 μl loading dye was also added into a well of the 2% agarose gel. Electrophoresis was carried out in 0.5X TBE buffer on the gel at a constant voltage of 100 volts for about 1.5 hours. Following electrophoresis, the gel was stained with 1 μg/ml ethidium bromide solution for 3 minutes and then destained with deionized water for 20 minutes. The stained gel was visualized and analyzed by the Bio-Rad Gel Doc 2000 under UV illumination.

The gel images were captured and analyzed by the ImageQuant software (Molecular Dynamics) to quantify the intensity of each gel band. The relative intensity of each band was compared with the solvent control following normalization with respect to the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### 2.2.13 Protein Expression Study

There were 4 steps in studying protein expression:

#### 1) Treatment of Cells and Extraction of Proteins

WEHI-3B JCS cells ($10^4$ cells/ml) were treated with CLA-mix or CLA isomer for different periods of time at 37°C in a humidified 5% $CO_2$ incubator. The cells were harvested and washed twice with cold PBS by centrifuging at 430 × g for 5 minutes at 4°C. The pellet was then resuspended with lysis buffer (50 μl lysis buffer/$10^6$ cells) and vortexed vigorously. Afterwards, the cell lysates were kept on ice for at least 30 minutes and centrifuged at 20,800 × g for 5 minutes at 4°C. The supernatants were collected for further assay.

#### 2) Quantification of Proteins

The protein concentrations of the cell lysates were determined by Bradford protein assay. Bovine serum albumin (BSA) solutions at 2 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml, and 10 μg/ml, prepared in deionized water in duplicate with 2 mg/ml BSA stock solution, were used as standards for the assay. Protein samples (1.6 μl) were diluted with 798.4 μl deionized water. Then 800 μl BSA standards or protein samples were mixed well with 200 μl Bradford reagent by inverting the cuvettes. After mixing, they were allowed to stand at room temperature for 5 minutes and the absorbance at 595 nm was measured using the Bradford reagent as a blank. A standard curve was constructed by the BioPhotometer (Eppendorf) in order that the concentrations of the protein samples could be determined by the machine automatically. Adjustments were made according to the measurement results by diluting the samples with lysis buffer, as a result, all the samples contained 20 μg proteins in a similar volume (~ 5 μl). Each protein sample (20 μg) was subjected to SDS-polyacrylamide gel electrophoresis

(PAGE).

### 3) Western Blotting Analysis

SDS-PAGE was employed to separate proteins according to their molecular weights. Five microlitres protein sample (~ 20 μg) was mixed with 5 μl 4X protein loading buffer, together with 10 μl lysis buffer to make up the total volume of 20 μl. The mixed protein samples were boiled for 5 minutes and loaded onto the SDS-polyacrylamide gel with 5% stacking gel. Kaleidoscope prestained protein standards (8 μl) were also loaded and the gel was run under constant voltage of 100 volts for about 2 hours. The percentage of acrylamide added in the running gel was varied in accordance with the molecular weight of the target protein. A protein of higher molecular weight needed a lower percentage of acrylamide gel and vice versa. With respect to the experimental results illustrated in this thesis, only 10% and 15% gels were used, and Table 2.4 shows the composition of these two percentages of SDS-polyacrylamide gel.

**Table 2.4: Composition of the SDS-polyacrylamide gel.**

| | 5 % Stacking Gel (3 ml) | Separating Gel (5 ml) | |
|---|---|---|---|
| | | 10% | 15% |
| Deionized water | 1.677 ml | 2 ml | 1.15 ml |
| Lower Buffer | ----- | 1.25 ml | 1.25 ml |
| Upper Buffer | 0.75 ml | ----- | ----- |
| 30% Acrylamide Stock | 0.5 ml | 1.65 ml | 2.5 ml |
| 10% SDS | 0.03 ml | 0.025 ml | 0.025 ml |
| 10% APS | 0.04 ml | 0.075 ml | 0.075 ml |
| TEMED | 3 µl | 2 µl | 2 µl |

Following electrophoresis, the stacking gel was cut with a spacer and discarded. The gel was put into deionized water for 5 minutes to remove SDS that could hamper the protein transfer, and then washed twice in the transfer buffer for 5 minutes. A piece of PVDF membrane was activated with 100% methanol and soaked in 1X transfer buffer, together with 6 pieces of filter paper (3 MM Whatman chromatography papers) for 10 minutes. Afterwards, 3 pieces of filter paper were placed on the semi-dry electroblotter (Bio-Rad Laboratories), followed by a piece of PVDF membrane. The gel was then placed carefully onto the membrane and lastly 3 more pieces of filter paper were covered onto the gel. Air bubbles were excluded by rolling a glass tube on the surface of filter papers and the surrounding water should be dried before electroblotting. The proteins were transferred from the gel onto the PVDF membrane after applying voltage at 16 volts for 35 minutes. After electroblotting, the membrane was washed with 1X washing buffer for 10 minutes, and then incubated with 5% skimmed milk at room temperature for at least 1 hour

with rotation to block the non-specific sites for probing. Primary antibody probing the target protein was added into the milk solution at a ratio of 1:1,000. The membrane was incubated with the primary antibody overnight at 4°C with rotation. On the next day, the primary antibody was removed and the membrane was washed thrice with 1X washing buffer for 5-10 minutes. The membrane was then incubated with the corresponding horseradish peroxidase-linked secondary antibody at 1:1,000 dilution in washing buffer for 1 hour at room temperature with rotation. After incubation, the membrane was washed thrice with 1X washing buffer and then subjected to ECL assay.

**4) ECL Assay**

Two detection reagents (reagent A and reagent B, 0.5 ml each) were mixed and poured onto the membrane. Excess mixture was removed. The membrane was then placed in a transparent plastic bag and the ECL film was placed above it in dark room. The film cassette was closed for different periods of exposure time, varying from 15 seconds to 3 minutes. Finally the film was developed and the specific protein bands could be visualized on the X-ray film. Band intensity was quantified by the ImageQuant software (Molecular Dynamics). The relative intensity of each protein band was compared with the solvent control following normalization with respect to the house-keeping protein β-actin.

### 2.2.14 Measurement of Cell Differentiation

**1) Cell Morphology Study**

To assess the capability of conjugated linoleic acid to induce cellular differentiation, WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix at 37°C for 48 hours. The solvent control-treated cells

were harvested and resuspended in 1 ml RPMI medium supplemented with 10% HI-FBS, whereas the CLA-mix-treated cells were allowed to stand at room temperature for a while. This procedure permits a short period of cold shock for detaching the differentiated cells from the culture flasks after taking them out from the 37°C incubator. The cells were then harvested by a cell scalper or by washing them twice with cold plain RPMI with vigorously shaking. The harvested cells were resuspended in 1 ml RPMI + 10% HI-FBS. Cell morphology was then examined by the preparation of cytospin smears. CLA-mix-treated WEHI-3B JCS cells ($2x10^5$ cells) were fixed onto a microscopic slide by cytocentrifugation at 500 rpm for 5 minutes using the Shandon Cytospin 3 centrifuge (Shadon Scientific Ltd., U.K.). The cells were then allowed to be air-dried. They were subsequently stained with each of the three different Hemacolor staining solutions for 15 seconds and destained under running tap water. Finally, the air-dried cells on the slides were mounted with neutral mounting medium, Canada Balsam (Sigma Chemical Co.) and the cell morphology was examined under the light microscope.

**2) Immunophenotyping**

WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 50 μM or 150 μM CLA-mix at 37°C for 48 hours. The untreated cells were harvested and resuspended in 1 ml RPMI + 10% HI-FBS, whereas the CLA-mix-treated cells were allowed to stand at room temperature for a while. The cells were then harvested by a cell scalper or by washing them twice with cold plain RPMI with vigorous shaking. The harvested cells were resuspended in 1 ml RPMI + 10% HI-FBS and cell count was performed with the hemocytometer. WEHI-3B JCS cells ($2.5x10^5$) were added to a transparent conical centrifuge tube containing 10 ml FACS medium and spun at 405 x g for 5 minutes. The supernatant was removed by aspiration and the pellet was suspended in a final volume

of 0.2 ml FACS medium. Rat IgG (10 μg/ml) and mouse IgG (10 μg/ml) were then added to the cell suspension to block the Fc receptors. After incubation at 4°C for 30 minutes with occasional shaking, the cells were washed twice with the FACS medium. The pellet was resuspended in a final volume of 50 μl FACS medium and dispended into each well of a 96-well U-bottomed microtiter plate (~2 x $10^5$ cells / 50 μl / well). Fifty microlitres primary antibody or PBS was added to each well. After incubation at 4°C for 30 minutes with occasional shaking, the cells were washed twice with FACS medium by spinning at 304 x g for 3 minutes at 4°C. FITC-conjugated sheep anti-rat Ig (1 μg) was then added to each well. After incubation at 4°C for 30 minutes (with occasional shaking), the cells were washed thrice with FACS medium by spinning at 304 x g for 3 minutes at 4°C. The pellets were then resuspended in 0.6 ml FACS fixative in FACS tubes. FACS analysis was performed.

### 3) Measurement of Activity of Monocytic Serine Esterase Activity

WEHI-3B JCS cells ($10^4$ cells/ml) were incubated respectively with the solvent control, 100 μM or 150 μM CLA-mix at 37°C for 72 hours. The solvent control-treated cells were harvested and resuspended in 1 ml RPMI + 10% HI-FBS, whereas the CLA-mix-treated cells were allowed to stand at room temperature for a while. The cells were then harvested by a cell scalper or by washing them twice with cold plain RPMI with vigorously shaking. The harvested cells were resuspended in 1 ml RPMI + 10% HI-FBS for cell counting. They were then resuspended in RPMI + 10% HI-FBS with a cell density of 2x$10^5$ cells/well in 200 μl. The cell suspension was then dispensed into a 96-well U-bottomed microtiter plate. Then 20 μl fluorescein diacetate (200 μg/ml) was added into each well. The samples were incubated at room temperature in dark for 30 minutes with occasional shaking. The cells were washed

twice with FACS medium by spinning at 304 x g for 3 minutes at 4°C. The cell pellets were resuspended in 0.8 ml FACS fixative in FACS tubes and kept in dark. FACS analysis was performed.

**4) Assay for Endocytosis**

WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with the solvent control, 100 μM or 150 μM CLA-mix at 37°C for 72 hours. The solvent control-treated cells were harvested and resuspended in 1 ml RPMI + 10% HI-FBS, whereas the CLA-mix-treated cells were allowed to stand at room temperature for a while. The cells were then harvested by a cell scalper or by washing them twice with cold plain RPMI with vigorously shaking. The harvested cells were resuspended in 1 ml RPMI + 10% HI-FBS for cell counting. They were then resuspended in a 15 ml conical tissue culture tube containing 0.9 ml RPMI + 10% HI-FBS. Then 0.1 ml FITC-conjugated BSA (1 mg/ml) was also added to the cells, which were incubated at 37°C in dark for 6 hours, with occasional shaking for every 30 minutes. The cells were subsequently washed thrice by spinning at 405 x g for 5 minutes, and were fixed with 1 ml FACS fixative in FACS tubes. FACS analysis was performed.

### 2.2.15 Statistical Analysis

Each experiment was performed at least two to three times and the results of only one representative experiment were presented. The data were expressed as arithmetic mean ± standard error (S.E.) of triplicate or quadruplicate determinations performed under the same conditions. The student's t-test was used for statistical analysis. Normally, $p < 0.05$ was regarded as significantly different.

# CHAPTER 3

# STUDIES ON THE ANTI-TUMOR ACTIVITY OF CONJUGATED LINOLEIC ACID ON MYELOID LEUKEMIA CELLS

# 3.1 Introduction

CLA has received considerable attention in recent years because it has been shown to promote various beneficial health-related effects in animals, as discussed in Chapter 1, particularly its anti-carcinogenic and anti-tumor activities. Recently, CLA has been reported to exhibit growth-inhibitory effect on a variety of human tumor cell lines derived from bladder (639V and SG65), breast (MCF7 and MDA-MB-231), colorectal region (HT-29 and MIP-101), liver (hepatoma HepG2 and SK-HEP-1), nervous system (glioblastoma ADF), prostate (LNCaP and PC-3), and stomach (SGC-7901) (Palombo *et al.*, 2002; Chen *et al.*, 2003; Maggiora *et al.*, 2004). Apart from *in vitro* studies, however, the majority of CLA researches has been carried out on its chemopreventive effect on mammary and colon tumorigenesis *in vivo* (Liew *et al.*, 1995; Visonneau *et al.*, 1997; Futakuchi *et al.*, 2002). By contrast, dietary CLA did not influence the growth of pancreatic solid tumor in Syrian hamsters (Kilian *et al.*, 2003). To date, few data are available to show the anti-tumor activity of CLA on other kinds of tumor cells (Maggiora *et al.*, 2004).

In this study, the anti-proliferative activity of a mixture of CLA isomers, so-called CLA-mix, and some of its isomers on the murine myelomonocytic leukemia WEHI-3B JCS cells was investigated. The growth-inhibitory effect of CLA-mix was also determined using a variety of murine and human myeloid leukemia cell lines, and its cytotoxic effect on the leukemia cells as well as normal myeloid cells such as murine peritoneal macrophages was also examined. Moreover, the kinetics and the reversibility of CLA-mix with regard to its anti-proliferative activity on the WEHI-3B JCS cells were studied. In addition to these *in vitro* studies, the ability of CLA-mix to

suppress the *in vivo* tumorigenicity of WEHI-3B JCS cells in syngeneic BALB/c mice was also assessed. Furthermore, the action mechanisms by which CLA-mix and the *trans*-9, *trans*-11 CLA (9E, 11E-CLA) isomer could exert their *in vitro* anti-proliferative activity on the WEHI-3B JCS cells were examined. The cell cycle profiles of the CLA-mix- and 9E, 11E-CLA-treated WEHI-3B JCS cells, and their expression of certain cell cycle-regulatory genes were studied using flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR) respectively.

# 3.2 Results

## 3.2.1 Anti-proliferative Activity of CLA-mix and CLA Isomers on Various Myeloid Leukemia Cell Lines *In Vitro*

In the initial experiments, the anti-proliferative activity of a mixture of CLA isomers (CLA-mix) on the murine myelomonocytic leukemia WEHI-3B JCS cells was evaluated by the standard colorimetric MTT assay as described in Chapter 2. The WEHI-3B JCS cells ($10^4$ cells/ml) were cultured with different concentrations of CLA-mix for 48 hours. During the last 2-3 hours of incubation, the cells were co-cultured with the MTT salt. In fact, the MTT assay measures the active metabolism of the mitochondrial dehydrogenases of living cells which can reflect the viable cell number of the WEHI-3B JCS cells following treatment with CLA-mix. In addition to the WEHI-3B JCS cell line, the anti-proliferative effect of CLA-mix was also examined using a variety of murine and human myeloid leukemia cell lines, including M1, HL-60, NB4, and K-562, and also the human lymphoma U-937 cells. Moreover, the growth-inhibitory effect of CLA-mix was compared with its parental fatty acid, linoleic acid (LA), and its four isomers, namely *cis*-9, *trans*-11 CLA (9Z, 11E-CLA), *trans*-10, *cis*-12 CLA (10E, 12Z-CLA), *cis*-9, *cis*-11 CLA (9Z, 11Z-CLA), and *trans*-9, *trans*-11 CLA (9E, 11E-CLA). It was found that the growth of WEHI-3B JCS cells was inhibited dose-dependently by CLA-mix (Fig. 3.1). The observed growth-inhibtory effect of CLA-mix on WEHI-3B cells was not due to the solvent effect, as the solvent control (ethanol), even at the highest concentration (0.1% v/v) used in my studies, exhibited little, if any, growth-inhibitory effect on the WEHI-3B JCS cells after 48 hours of incubation (Fig. 3.1). Similar growth-inhibitory effect of CLA-mix was also demonstrated in various murine and human hematologic malignant

cell lines (Fig. 3.2). Table 3.1 shows the estimated $IC_{50}$ values of CLA-mix on various leukemia or lymphoma cell lines. By comparing these cell lines, the human promyelocytic leukemia NB4 cell line and the murine myelomonocytic leukemia WEHI-3B JCS cell line were found to be most sensitive to the anti-proliferative activity of CLA-mix. Nevertheless, owing to its short doubling time and its well-characterized features, the WEHI-3B JCS cell line was chosen as the major cell model for further studies. In addition, the WEHI-3B JCS cells can grow in syngeneic BALB/c mice, thus facilitating *in vivo* studies without stimulating immune rejections.

When compared to its parental fatty acid, LA (Fig. 3.3), and all of the four CLA isomers being investigated (Fig. 3.4), CLA-mix was found to be the most potent growth-inhibitor. A comparison of the sensitivity of WEHI-3B JCS cells to CLA-mix and its four isomers was shown in Table 3.2. Specifically, among all CLA isomers being studied, the 9E, 11E-CLA, 10E, 12Z-CLA and 9Z, 11E-CLA inhibited the growth of WEHI-3B JCS cells to a similar extent while the 9Z, 11Z-CLA was found to be slightly weaker, especially at 200 μM concentration. Since CLA-mix inhibited the growth of WEHI-3B JCS cells to a greater extent than each of its isomers, it is possible that interactions might occur among individual CLA isomers, resulting in higher anti-proliferative activity on WEHI-3B JCS cells following exposure to CLA-mix.

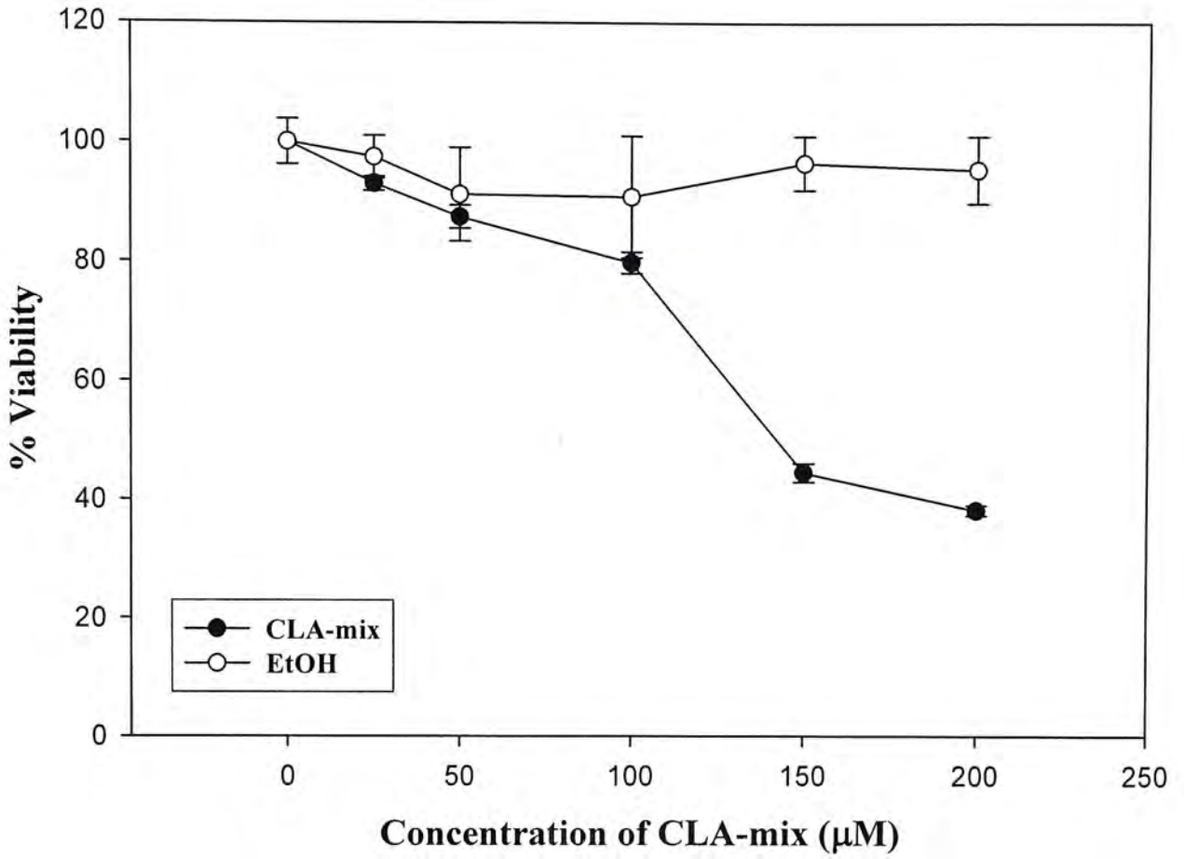



**Fig. 3.1: Growth-inhibitory effect of CLA-mix on the murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix (0-200 μM) and ethanol (EtOH) (0-0.1%) at 37°C for 48 hours. Cell proliferation was determined by the MTT assay as described in Chapter 2. Results were expressed as % viability using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

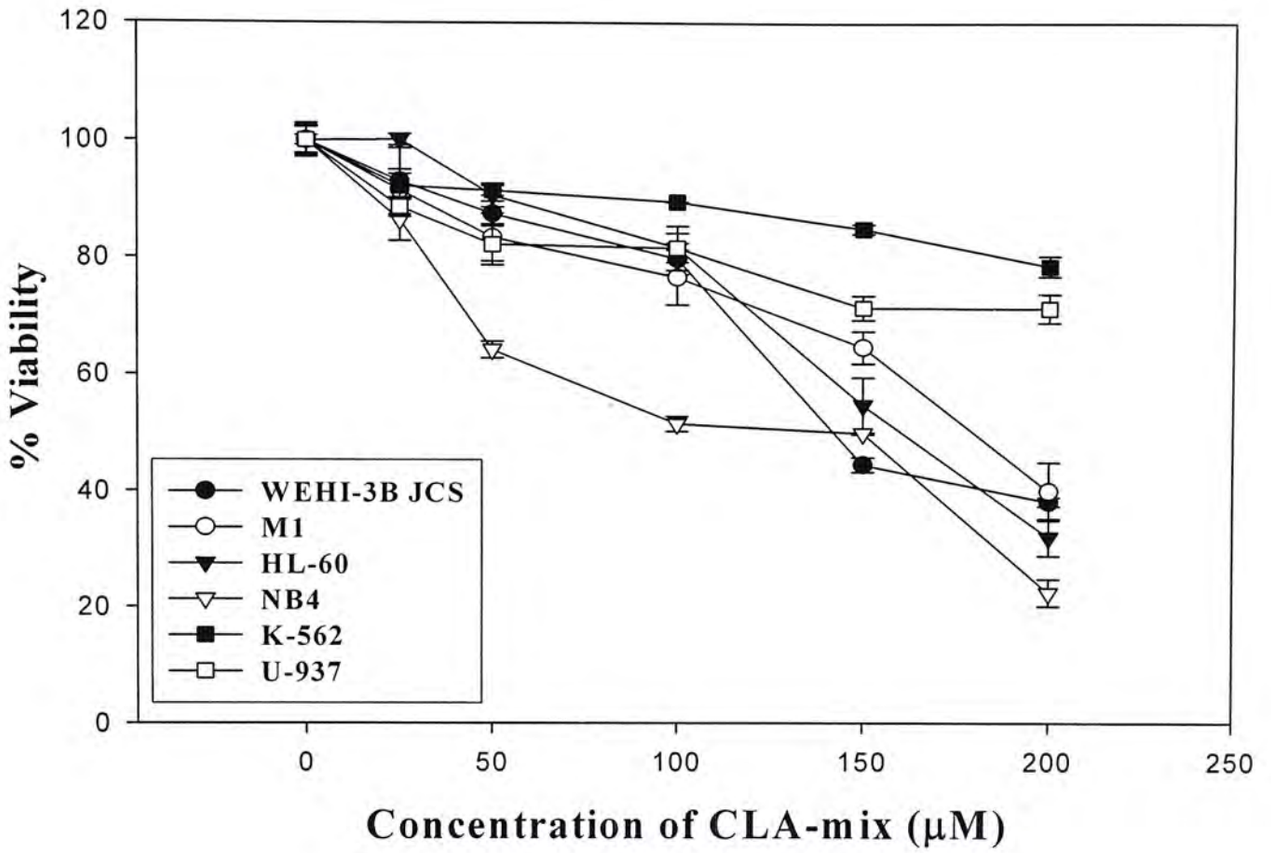



**Fig. 3.2: Growth-inhibitory effect of CLA-mix on various types of murine and human leukemia or lymphoma cell lines as determined by the MTT assay.** Leukemia cells were incubated with different concentrations of CLA-mix at 37°C for 48 hours. Cell proliferation was determined by the MTT assay as described in Chapter 2. Results were expressed as % viability using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

**Table 3.1: The estimated $IC_{50}$ values of CLA-mix on various types of leukemia or lymphoma cell lines.**

| Tumor cell line | | Estimated $IC_{50}$ value (μM) |
|---|---|---|
| Murine myelomonocytic leukemia | WEHI-3B JCS | ~ 150 |
| Murine acute myeloblastic leukemia | M1 | ~ 180 |
| Human promyelocytic leukemia | HL-60 | ~ 160 |
| | NB4 | ~ 140 |
| Human chronic myelogenous leukemia | K-562 | > 200 |
| Human histiocytic monoblast-like lymphoma | U-937 | > 200 |

**$IC_{50}$ value is the estimated concentration of CLA-mix which can cause 50% inhibition on the *in vitro* growth of leukemia or lymphoma cells under specified experimental conditions.**

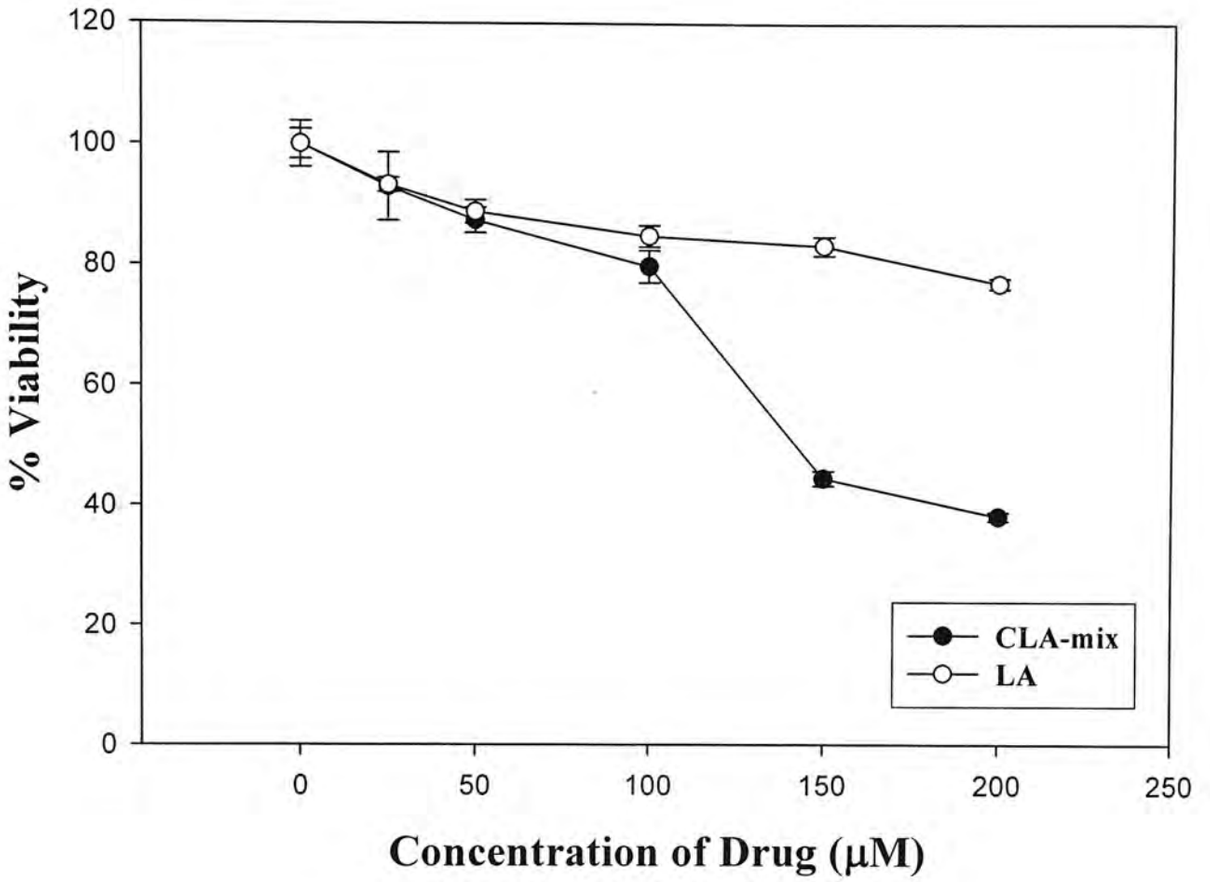



**Fig. 3.3: Growth-inhibitory effect of CLA-mix and LA on the murine myelomonocytic leukemia WEHI-3B JCS cells as determined by the MTT assay.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix and LA at 37°C for 48 hours. Cell proliferation was determined by the MTT assay as described in Chapter 2. Results were expressed as % viability using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

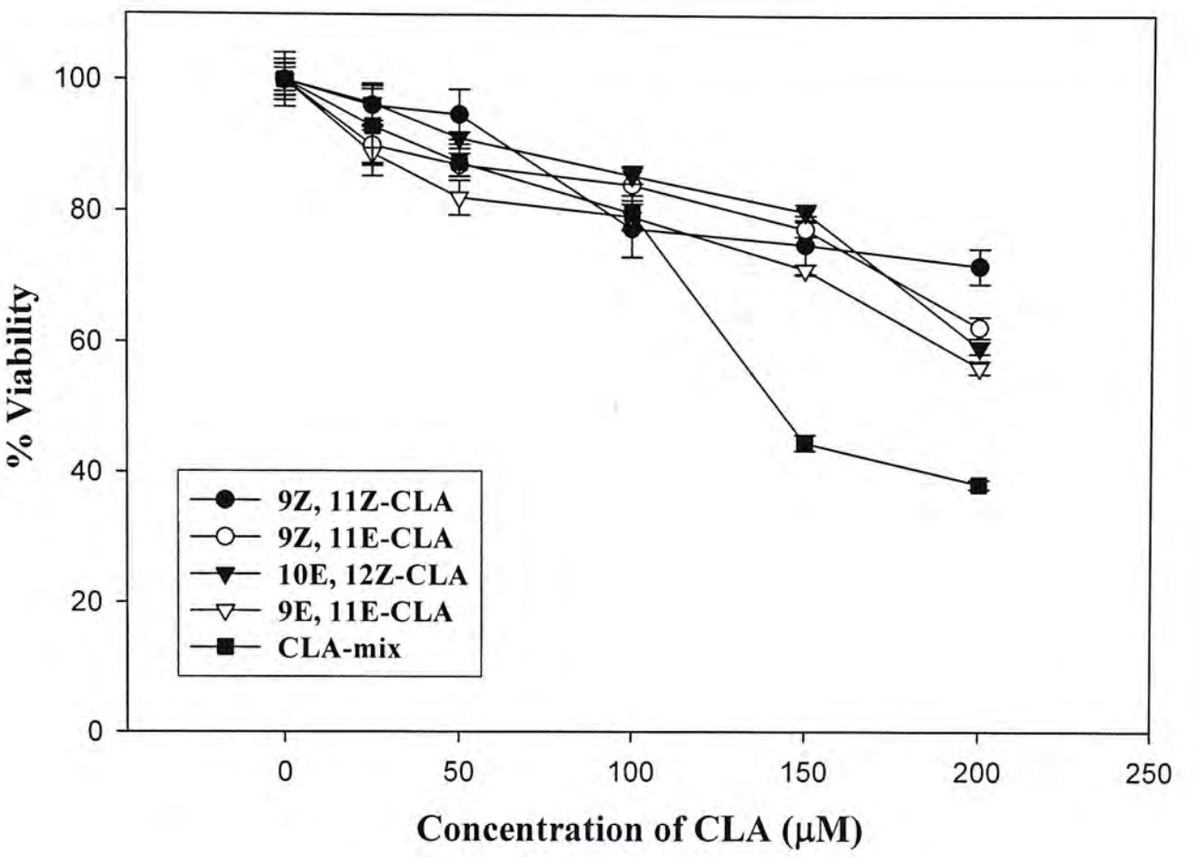



**Fig. 3.4: Growth-inhibitory effect of CLA-mix and its isomers on the murine myelomonocytic leukemia WEHI-3B JCS cells as determined by the MTT assay.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix and CLA isomers at 37°C for 48 hours. Cell proliferation was determined by the MTT assay as described in Chapter 2. Results were expressed as % viability using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

**Table 3.2: The estimated $IC_{50}$ values of CLA-mix and its isomers on the murine myelomonocytic leukemia WEHI-3B JCS cells.**

| Type of CLA | Estimated $IC_{50}$ value (μM) |
|---|---|
| CLA-mix | ~ 150 |
| 9Z, 11E-CLA | > 200 |
| 10E, 12Z-CLA | > 200 |
| 9Z, 11Z-CLA | > 200 |
| 9E, 11E-CLA | > 200 |

**$IC_{50}$ value is the estimated concentration of CLA-mix or its isomers which can cause 50% inhibition on the *in vitro* growth of the murine myelomonocytic leukemia WEHI-3B JCS cells under specified experimental conditions.**

### 3.2.2 Cytotoxic Effect of CLA-mix on the WEHI-3B JCS Cells *In Vitro*

The cytotoxicity of CLA-mix on the murine myelomonocytic leukemia WEHI-3B JCS was examined by the trypan blue exclusion assay. Following incubation with CLA-mix for 24, 48, and 72 hours, it was found that CLA-mix, at concentrations ranged from 25 to 200 μM, exhibited no direct cytotoxicity on the WEHI-3B JCS cells (Fig. 3.5). Therefore, our findings indicate that the anti-proliferative activity of CLA-mix is unlikely to be due to the direct cytotoxicity of CLA-mix on the WEHI-3B JCS cells.

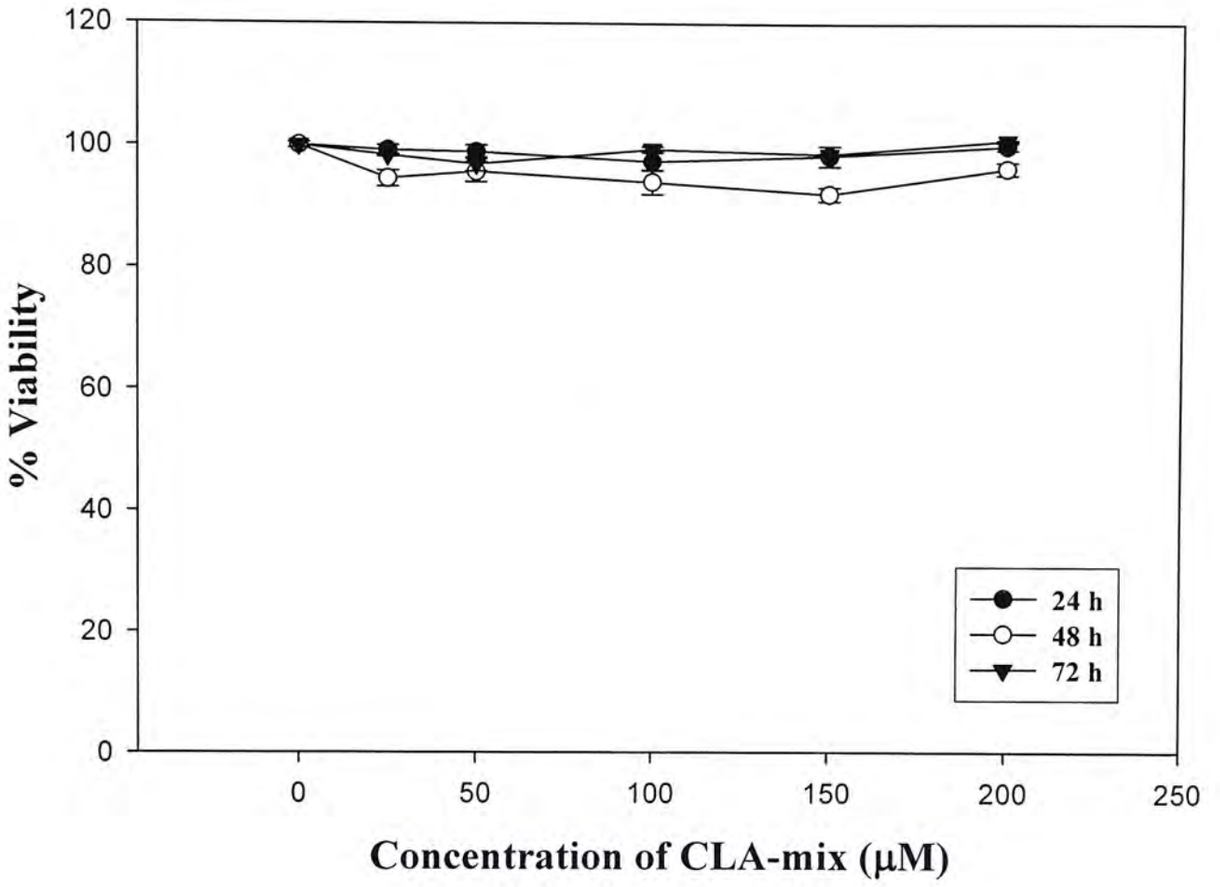



**Fig. 3.5: Effect of CLA-mix on the viability of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix at 37°C for 24, 48 and 72 hours. The numbers of viable and dead cells were then counted by using trypan blue exclusion assay. Results were expressed as % cell viability, using the untreated cells as a control. Each point represented the mean ± S.E. of triplicate cultures.

### 3.2.3 Cytotoxic Effect of CLA-mix on Primary Murine Myeloid Cells *In Vitro*

One of the major concerns for a novel medication to be applied clinically is its possible cytotoxic effect on the normal cells of our body. In order to examine whether CLA-mix exhibits any cytotoxic effect on normal cells, the thioglycollate-elicited murine peritoneal macrophages were cultured *in vitro* with different concentrations of CLA-mix at 37°C for 48 hours. The viability of the macrophages was then measured by the MTT assay. Figure 3.6 shows that CLA-mix exhibited only a slight cytotoxic effect on the macrophages over a wide range of concentrations (25-200 μM). The percentage of viable cells following CLA treatment was > 85%, even at the highest concentration tested. Moreover, when compared with its parental fatty acid, LA, it can be seen that CLA displayed less cytotoxic effect on the macrophages, suggesting that CLA-mix is relatively non-toxic to the normal cells such as murine peritoneal macrophages.

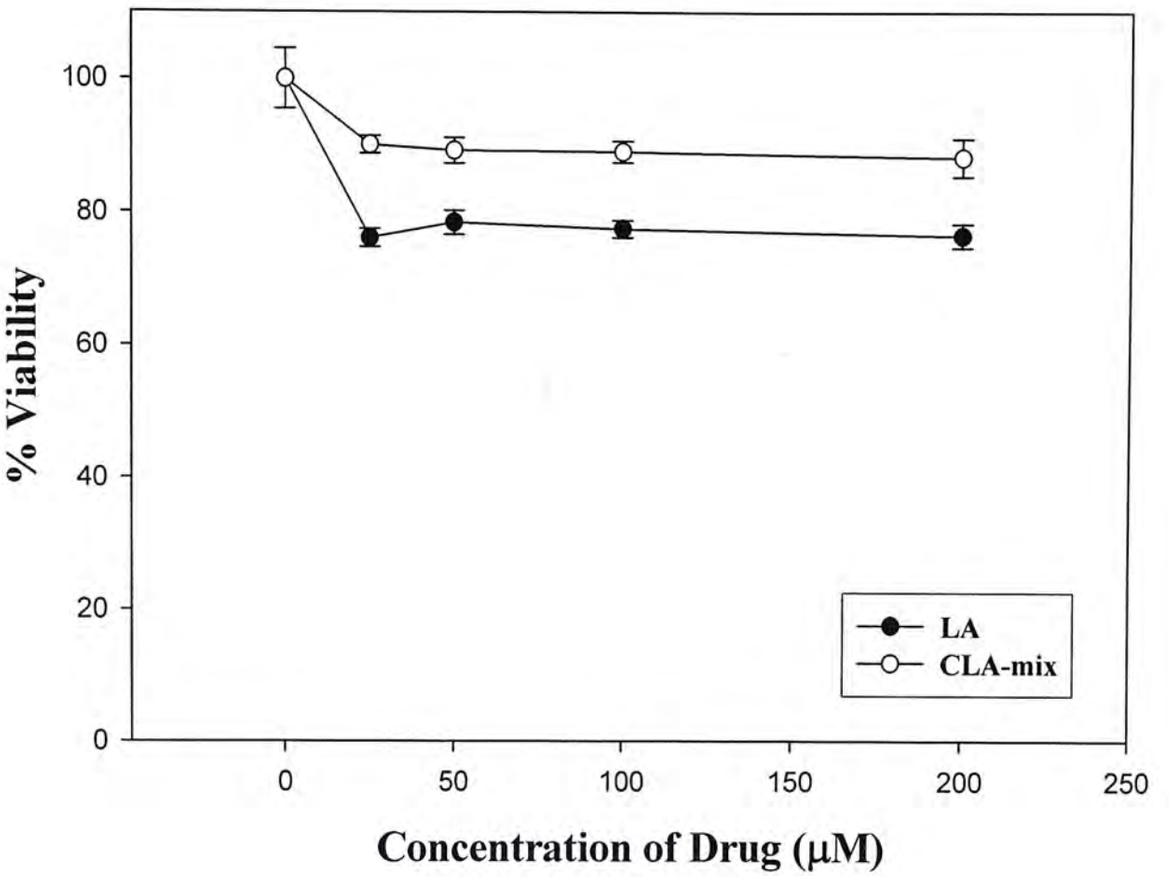



**Fig. 3.6: Effect of CLA-mix and linoleic acid (LA) on the viability of murine peritoneal macrophages *in vitro*.** Thioglycollate-elicited BALB/c peritoneal macrophages (5 x $10^6$ cells/ml) were cultured with different concentrations of CLA-mix and LA for 48 hours at 37°C. Cell proliferation was determined by the MTT assay as described in Chapter 2. Results were expressed as % viability using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

### 3.2.4 Kinetic and Reversibility Studies of the Anti-proliferative Activity of CLA-mix on the WEHI-3B JCS Cells

The tritiated thymidine ($^3$H-TdR) incorporation assay was also used to measure the anti-proliferative activity of CLA-mix on WEHI-3B JCS cells, as the incorporation of labeled DNA precursor, $^3$H-TdR, into the DNA of the cells is directly proportional to the extent of cell proliferation. In the present study, WEHI-3B JCS cells were incubated with different concentrations of CLA-mix (0 - 200 μM) at 37°C for 24, 48 and 72 hours and cell proliferation was then measured by the $^3$H-TdR incorporation assay. Figure 3.7 shows that CLA significantly inhibited the *in vitro* proliferation of WEHI-3B JCS cells in a dose- and time-dependent manner. The estimated $IC_{50}$ value at 48 hours of incubation is ~152 μM, which is very similar to the results obtained with the MTT assay (Fig. 3.1).

To test the reversibility of the anti-proliferative effect of CLA-mix on the WEHI-3B JCS cells, the leukemia cells were treated with CLA-mix at 150 μM and 200 μM for 6, 12, or 48 hours, then the CLA-mix was washed away by removing the medium and then replaced with complete medium and the cells were incubated up to 48 hours totally. As shown in Figure 3.8, the growth-inhibitory effect of CLA-mix at both concentrations was less than 25% after only 6 or 12 hours, but the % of inhibition reached up to 53% and 89% after 48-hour treatment with 150 μM and 200 μM CLA-mix, respectively. In general, the longer the exposure time to CLA-mix, the less reversibility the anti-proliferative effect was.

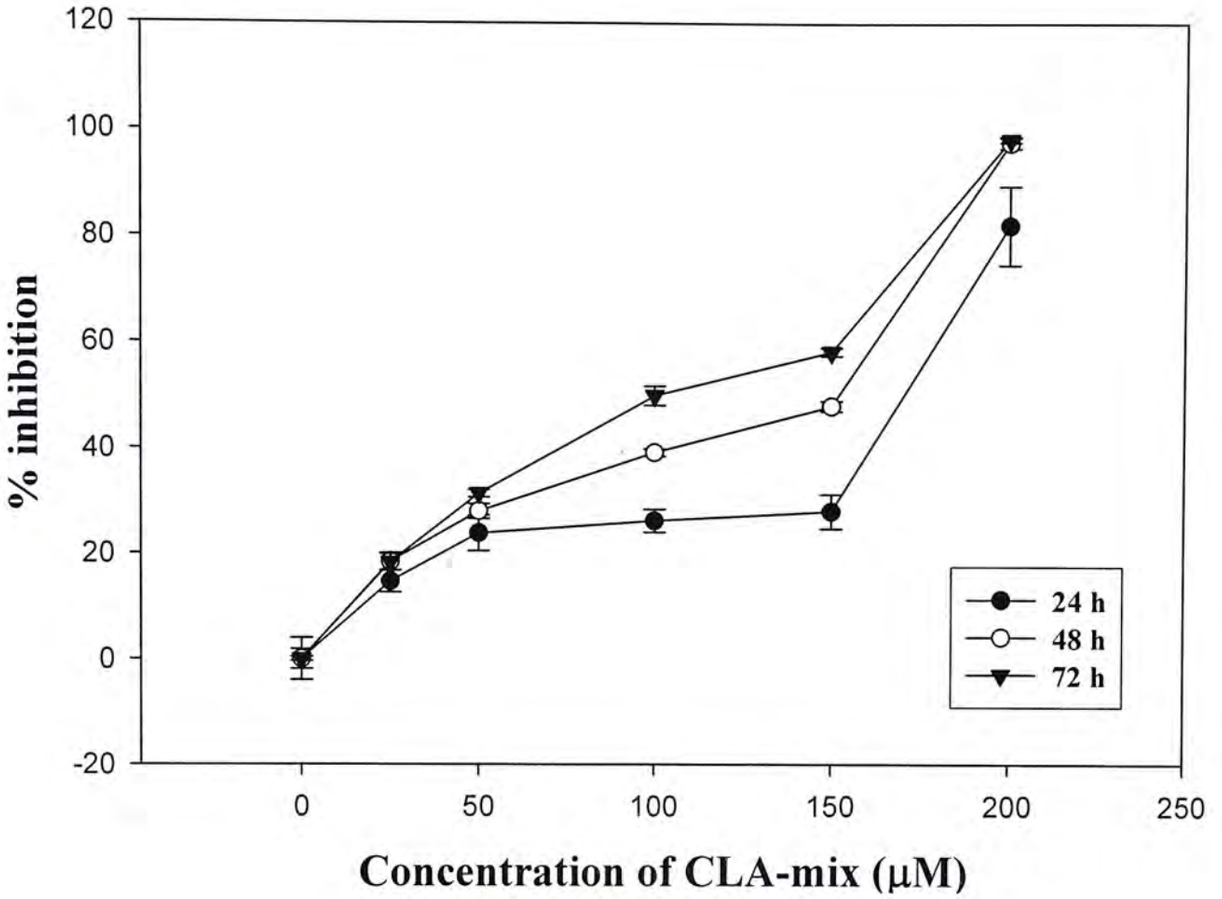



**Fig. 3.7: Kinetic study on the growth-inhibitory effect of CLA-mix on the murine myelomonocytic leukemia WEHI-3B JCS cells.** JCS cells ($10^4$ cells/ml) were incubated with different concentrations of CLA-mix at 37°C for different time intervals (24, 48 and 72 hours). Cultures were then pulsed with 0.5 μCi of $^3$H-TdR for 6 hours before harvest. Radioactivity, expressed as counts per minute (cpm), was determined using a liquid scintillation counter. Results were expressed as % inhibition of $^3$H-TdR incorporation with respect to the untreated control cells. Each point represents the mean ± S.E. of quadruplicate cultures.

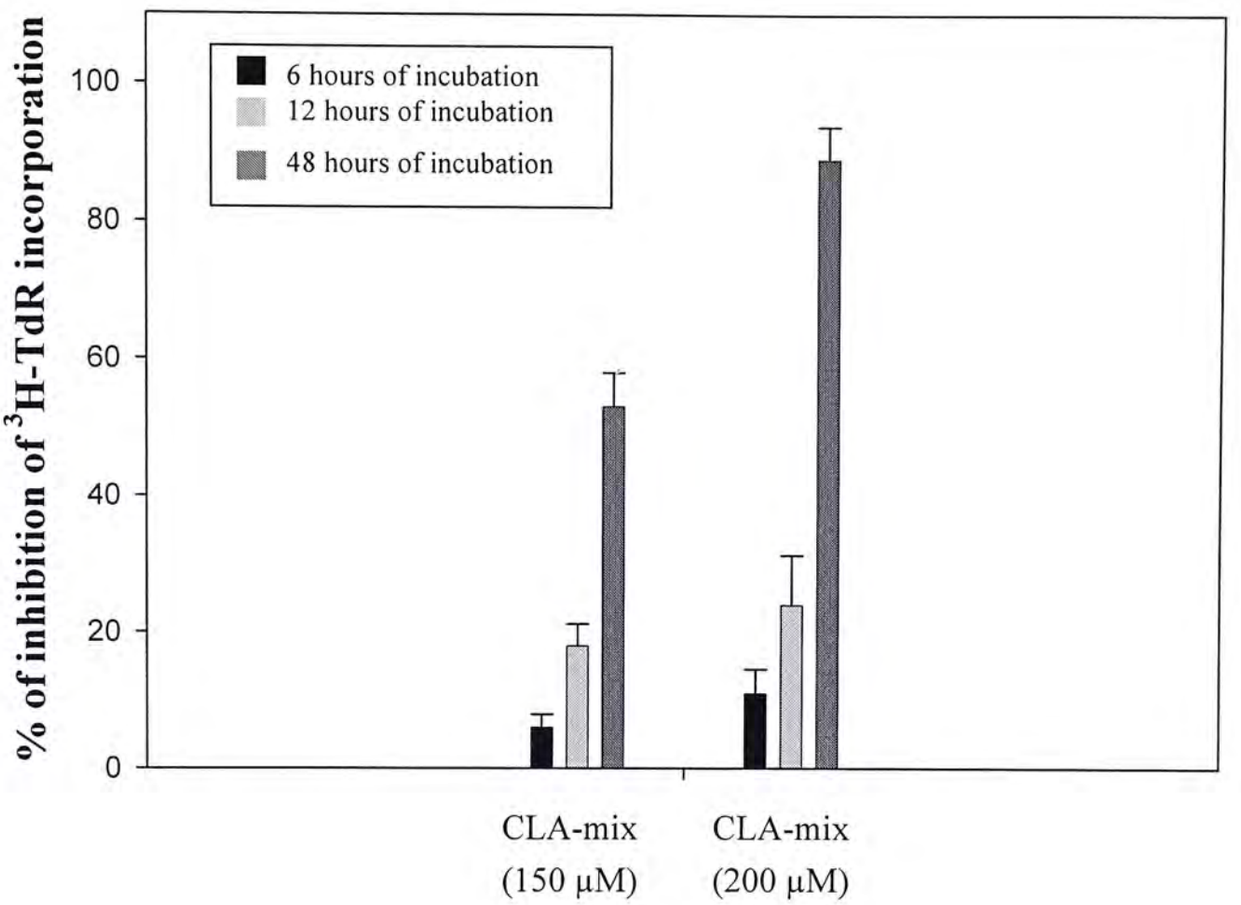


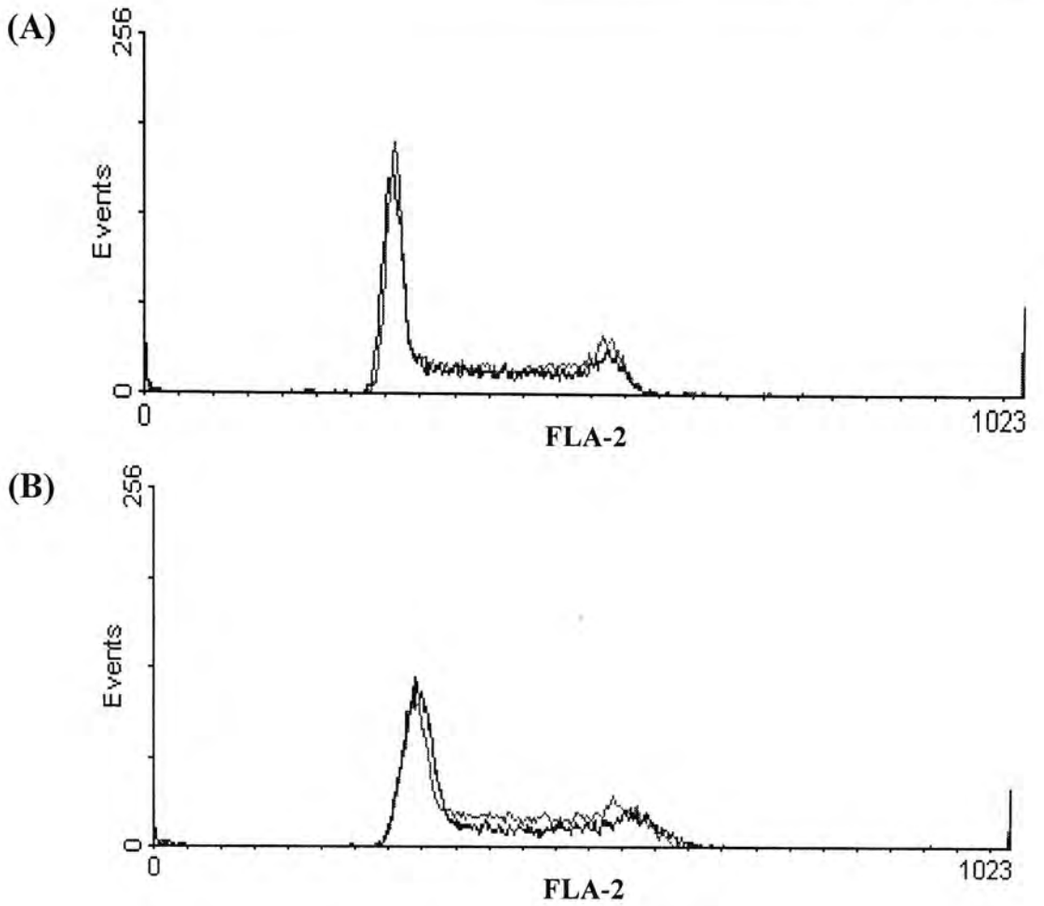

**Fig. 3.8: Reversibility study on the anti-proliferative effect of CLA-mix on the murine myelomonocytic leukemia WEHI-3B JCS cells.** JCS cells ($10^4$ cells/ml) were co-incubated with 150 μM or 200 μM CLA-mix at 37 °C for 6, 12, and 48 hours. CLA-mix were washed away and replaced by complete medium at the corresponding times and all cultures were incubated up to 48 hours. Cultures were then pulsed with 0.5 μCi of $^3$H-TdR for 6 hours before harvest. Radioactivity, expressed as counts per minute (cpm), was measured using a liquid scintillation counter. Results were expressed as % inhibition of $^3$H-TdR incorporation, using the untreated cells as a control. Each point represents the mean ± S.E. of quadruplicate cultures.

### 3.2.5 Effect of CLA-mix and its isomers on the Cell Cycle Profiles of the WEHI-3B JCS Cells *In Vitro*

Cell proliferation entails DNA synthesis followed by nuclear division and cytoplasm partition to yield two daughter cells. Such a sequential routine is known as the "cell cycle" (Massague, 2004). The eukaryotic cell cycle is divided into four phases: $G_1$, S, $G_2$, and M. The gap $G_1$ phase is incorporated between nuclear division (M) and DNA synthesis (S) during which the cell integrates mitogenic or growth-inhibitory signals to make further decisions regarding whether to enter S phase for self-renewal or pause for differentiation or apoptosis. In addition to the above four phases, the term $G_0$ is used to describe the cell that has exited the cell cycle and become quiescent. In fact, deregulation of the normal cell cycle is regarded as the hallmark of cancer (Johnson and Walker, 1999). Nevertheless, numerous novel opportunities have been targeted on checkpoint controls of the cell cycle to develop new therapeutic strategies, including induction of checkpoint arrest leading to cytostasis and ultimately apoptosis, arrest of proliferating cells in various phases of the cell cycle which may sensitize them to treatment with other therapeutic agents such as radiation, and targeting specific regulatory mediators of the cell cycle through either ectopic expression or down-regulation of certain cell cycle-regulatory genes.

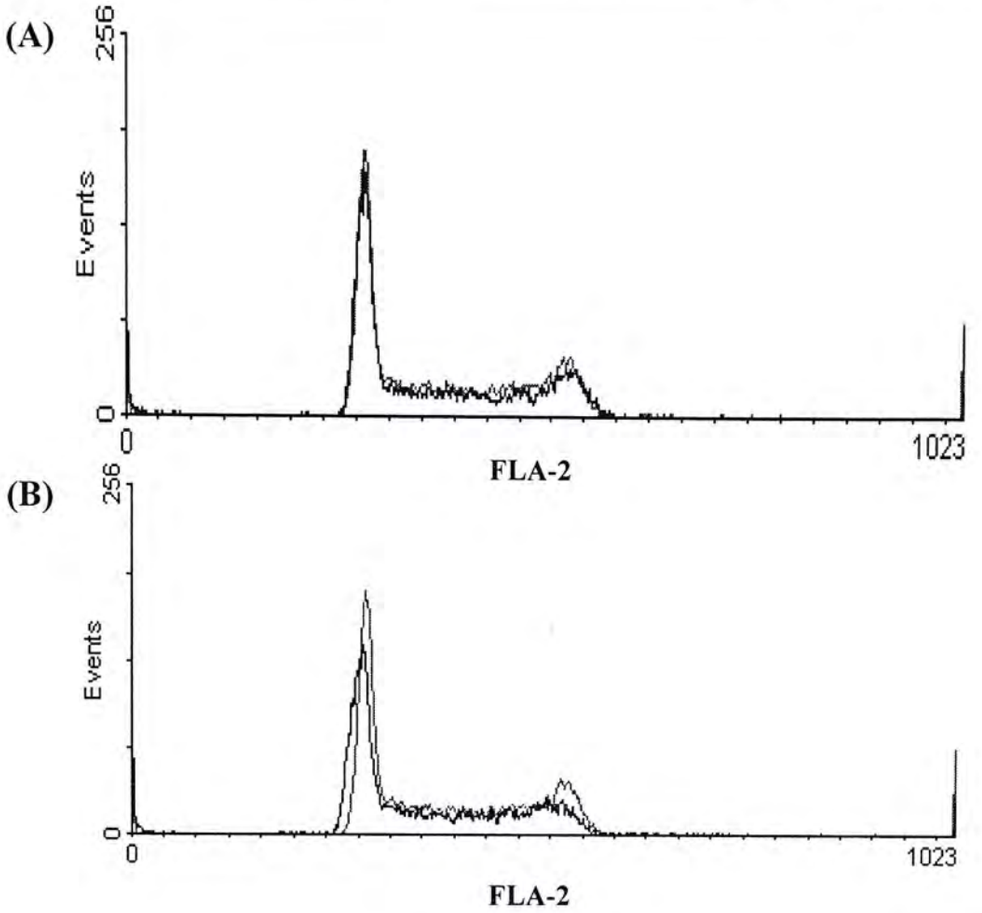
More recently, CLA-mix has been shown also to arrest the colon cancer HT-29 cells in the $G_1$ phase (Lim *et al.*, 2005). In the present study, we determined whether CLA-mix and its isomers inhibited proliferation of the murine myelomonocytic leukemia WEHI-3B JCS cells by alterating the cell cycle progression. For these experiments, two concentrations, 100 μM and 150 μM, of the CLA-mix and its

isomers were used. Our results showed that CLA-mix increased the percentage of cells in $G_0/G_1$ phase in a dose-dependent manner which was accompanied by a corresponding reduction in the percentages of cells in S phase (Fig. 3.9). Moreover, among the four CLA isomers being investigated, the 10E, 12Z-CLA increased the percentage of cells in $G_0/G_1$ phase to the greatest extent, though its effect was still less than CLA-mix (Fig. 3.10 - 3.13). Interestingly, the 9Z, 11Z-CLA isomer did not exert any effect on the cell cycle as there were no significant differences in the percentages of cells in each phase of the cell cycle when compared to the untreated control cells.



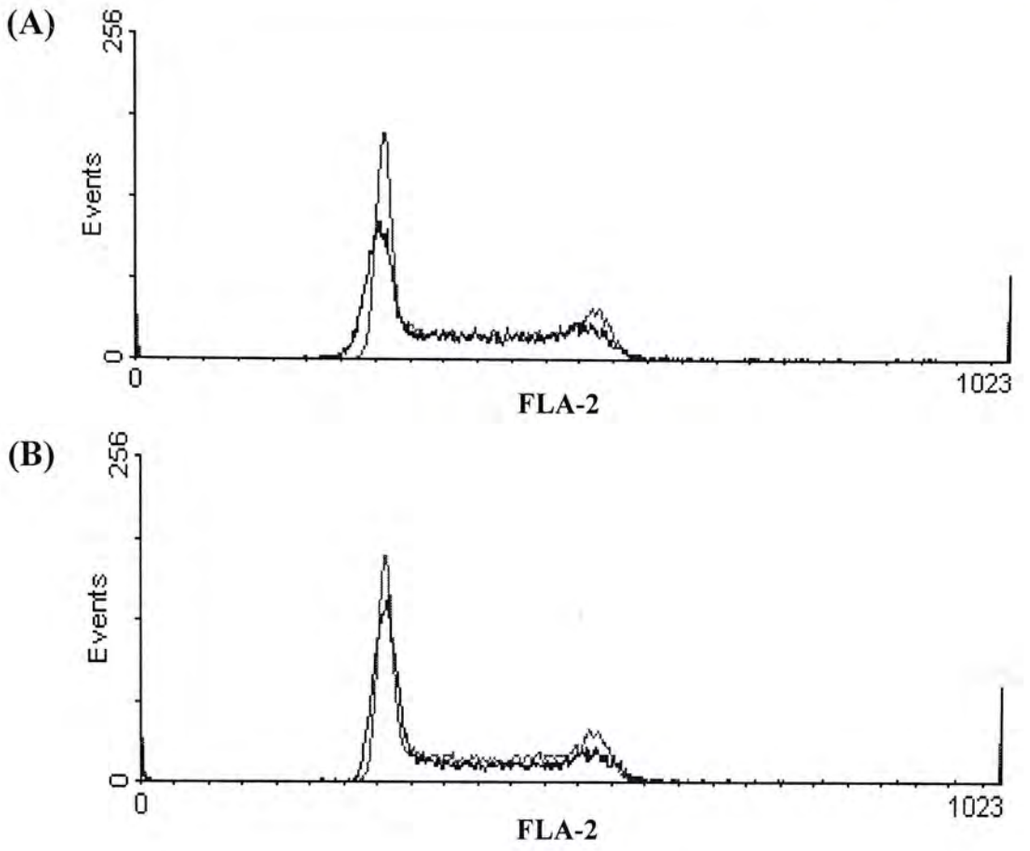

| | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| **Control cells** | 36.77 % | 51.14 % | 12.09 % |
| **CLA-mix-treated cells (100 µM)** | 48.66 % | 37.91 % | 13.43 % |
| **% Change** | + 11.89 % | - 13.23 % | + 1.34 % |
| **CLA-mix-treated cells (150 µM)** | 52.40 % | 31.22 % | 16.39 % |
| **% Change** | + 15.63 % | - 19.92 % | + 4.30 % |

**Fig. 3.9: Effect of CLA-mix on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 100 µM (A) or 150 µM (B) CLA-mix at 37°C for 24 hours. CLA-mix-treated cells ($10^6$ cells) were fixed with ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model.



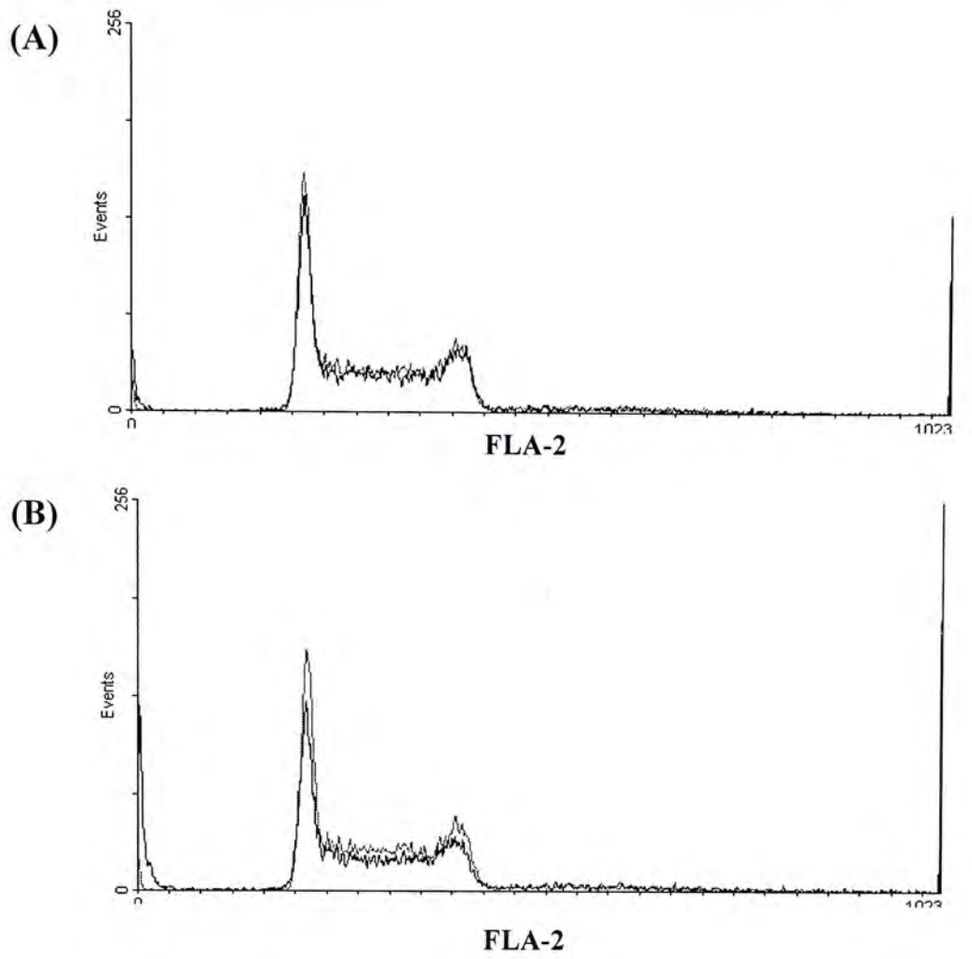

| | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| **Control** | 36.77 % | 51.14 % | 12.09 % |
| **9Z, 11E-CLA-treated cells (100 μM)** | 41.19 % | 47.28 % | 11.53 % |
| **% Change** | + 4.42 % | - 3.86 % | - 0.56 % |
| **9Z, 11E-CLA-treated cells (150 μM)** | 43.37 % | 46.91 % | 9.72 % |
| **% Change** | + 6.60 % | - 4.23 % | - 2.37 % |

**Fig. 3.10: Effect of 9Z, 11E-CLA on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 100 μM (A) or 150 μM (B) 9Z, 11E-CLA at 37°C for 24 hours. 9Z, 11E-CLA-treated cells ($10^6$ cells) were fixed with ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model.



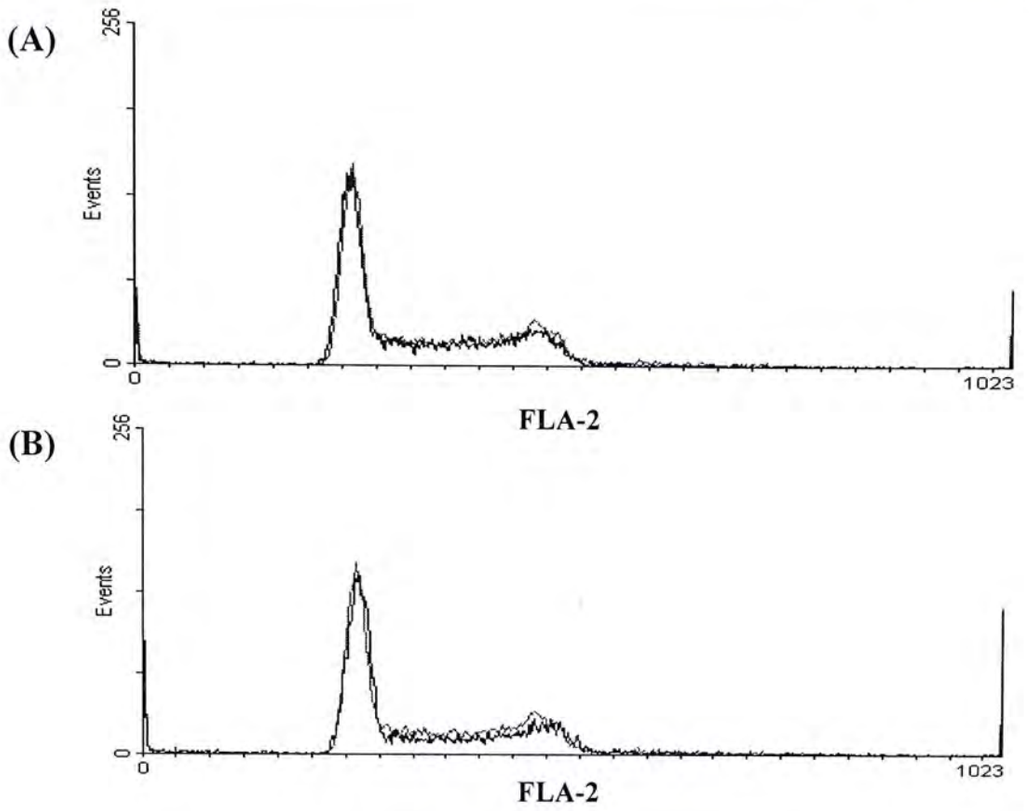

| | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| **Control** | 36.77 % | 51.14 % | 12.09 % |
| **10E, 12Z-CLA-treated cells (100 μM)** | 37.73 % | 50.44 % | 11.72 % |
| **% Change** | + 0.96 % | - 0.70 % | - 0.37 % |
| **10E, 12Z-CLA-treated cells (150 μM)** | 48.52 % | 41.37 % | 10.11 % |
| **% Change** | + 11.75 % | - 9.77 % | - 1.98 % |

**Fig. 3.11: Effect of 10E, 12Z-CLA on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 100 μM (A) or 150 μM (B) 10E, 12Z-CLA at 37°C for 24 hours. 10E, 12Z-CLA-treated cells ($10^6$ cells) were fixed with ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model.




| | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| **Control** | 28.67 % | 60.10 % | 11.23 % |
| **9Z, 11Z-CLA-treated cells (100 μM)** | 28.81 % | 58.96 % | 12.22 % |
| **% Change** | + 0.14 % | - 1.14 % | + 0.99 % |
| **9Z, 11Z-CLA-treated cells (150 μM)** | 28.70 % | 60.18 % | 11.12 % |
| **% Change** | + 0.03 % | - 0.08 % | - 0.11 % |

**Fig. 3.12: Effect of 9Z, 11Z-CLA on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 100 μM (A) or 150 μM (B) 9Z, 11Z-CLA at 37°C for 24 hours. 9Z, 11Z-CLA-treated cells ($10^6$ cells) were fixed with ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model.




| | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| **Control** | 42.49 % | 45.14 % | 12.37 % |
| **9E, 11E-CLA-treated cells (100 μM)** | 46.06 % | 43.22 % | 10.72 % |
| **% Change** | + 3.57 % | - 1.92 % | - 1.65 % |
| **9E, 11E-CLA-treated cells (150 μM)** | 51.26 % | 35.82 % | 12.93 % |
| **% Change** | + 8.77 % | - 9.32 % | + 0.56 % |

**Fig. 3.13: Effect of 9E, 11E-CLA on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with 100 μM (A) or 150 μM (B) 9E, 11E-CLA at 37°C for 24 hours. 9E, 11E-CLA-treated cells ($10^6$ cells) were fixed with ethanol and stained with PI under hypotonic conditions. PI-stained cells were analyzed for fluorescence intensity using the FACSort flow cytometer. Cell cycle distribution was calculated by the MODFIT program using RFIT analysis model.

### 3.2.6 Effect of CLA-mix and its isomer on the Expression of Cell Cycle-regulatory Genes in the WEHI-3B JCS Cells

From the previous section, it was found that the growth-inhibitory activity of CLA-mix was mediated, at least in part, through halting cell cycle progression at the $G_1$ phase. In addition, there were no previous reports documenting the growth-inhibitory effect and action mechanisms of the 9E, 11E-CLA isomer on leukemia cells, both *in vitro* and *in vivo*. Therefore, further examination on the expression of cell cycle-regulatory genes would be carried out in the CLA-mix-treated and the 9E, 11E-CLA-treated WEHI-3B JCS cells.

Upon DNA damage, it has been reported that the tumor suppressor protein p53 can either trigger cell cycle arrest in an attempt to repair the damage, or it can induce apoptosis to prevent the cell from developing into a tumor (Eastman, 2004). In fact, p53 is particularly important for regulating progression through $G_1$ phase of the cell cycle while other checkpoint regulators are responsible for arrest in S or $G_2$ phase. On the other hand, there is strong evidence to suggest that the cyclin-dependent kinase (cdk) inhibitor (CKI) $p21^{CIP1/WAF1}$ is the key mediator of the p53-regulated $G_1$ checkpoint control (Deng *et al.*, 1995). The $p21^{CIP1/WAF1}$ gene promoter contains a p53-binding site that allows p53 to transcriptionally activate the $p21^{CIP1/WAF1}$ gene (Johnson and Walker, 1999). Induction of $p21^{CIP1/WAF1}$ halts cell cycle progression by inhibiting a variety of cyclin/cdk complexes. Cdks allow progression through different phases of the cell cycle by phosphorylating specific substrates, and their kinase activity is dependent on the presence of activated cyclins. Nevertheless, the $G_1$/S-promoting cyclin/cdk complexes include cyclin D/cdk4/6, cyclin E/cdk2, and

cyclin A/cdk1/2 (King and Cidlowski, 1998; Field and Schley, 2004).

In the present study, the expression of certain cell cycle-regulatory genes was examined by the semi-quantitative RT-PCR technique. As shown in Fig. 3.14A and B, the mRNA levels of the *p53* gene were increased following treatment with CLA-mix and 9E, 11E-CLA for 12 and 6 hours, respectively. However, their effects on the *p53* gene expression were transient as the mRNA levels returned to normal at 24 hours in both CLA-mix-treated and 9E, 11E-CLA-treated WEHI-3B JCS cells. Moreover, both CLA-mix and 9E, 11E-CLA increased the expression of the $p21^{CIP1/WAF1}$ gene and decreased the expression of the *cyclin A* gene (Fig. 3.15 and 3.16). In addition to $p21^{CIP1/WAF1}$, CLA-mix also increased the mRNA levels of another CKI, the $p27^{KIP1}$ (Fig. 3.16), which can inhibit the cyclin/cdk complexes such as the cyclin A/cdk2 from promoting G1 to S phase transition (Russo *et al.*, 1996).

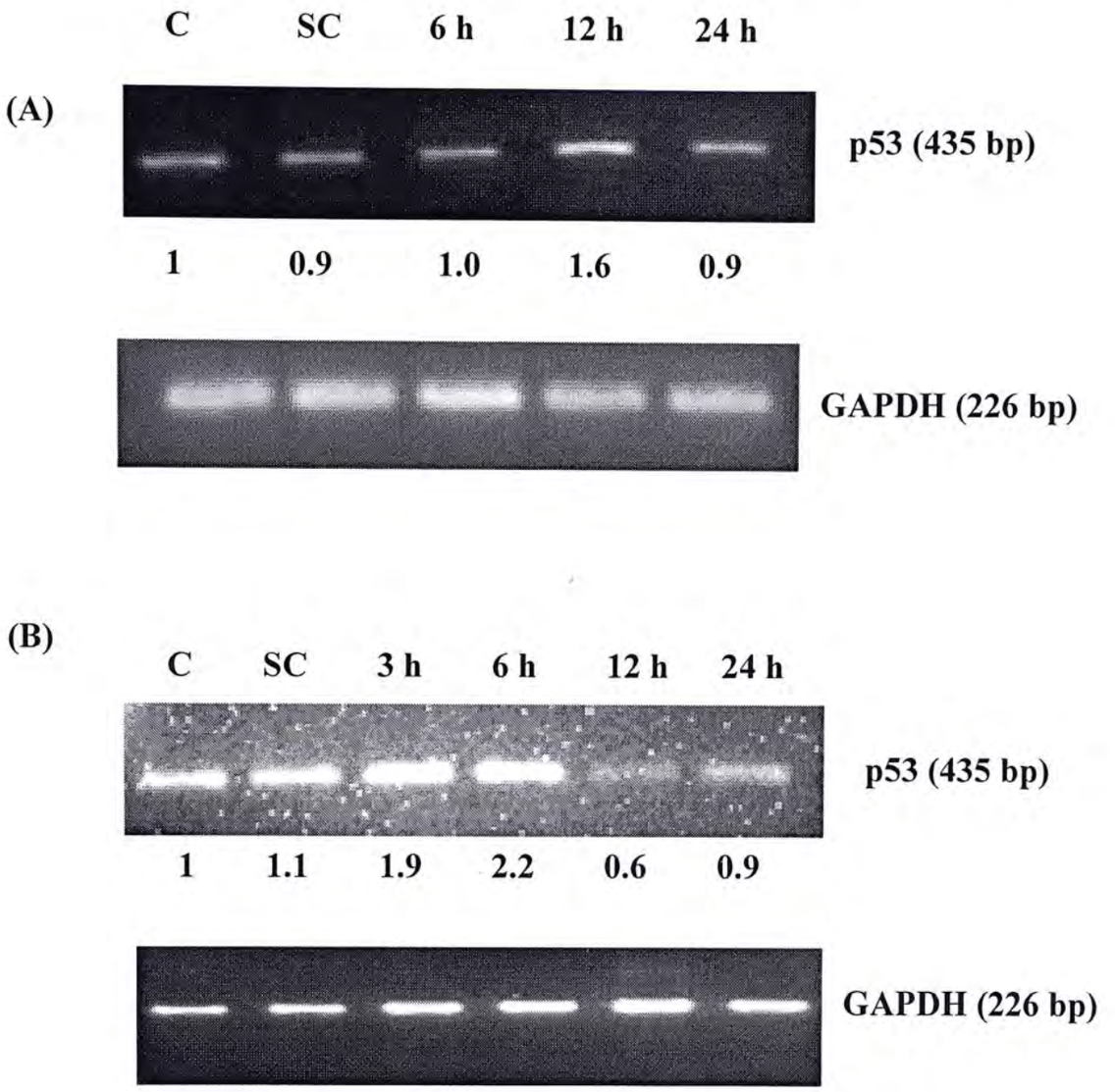



**Fig. 3.14: Analysis of the p53 gene expression in the (A) CLA-mix-treated and (B) 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by RT-PCR.** JCS cells ($10^6$ cells) were incubated with 150 μM of CLA at 37°C for different time intervals (3, 6, 12 and 24 hours) or treated with medium (C) or ethanol (SC) as controls. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding medium control.

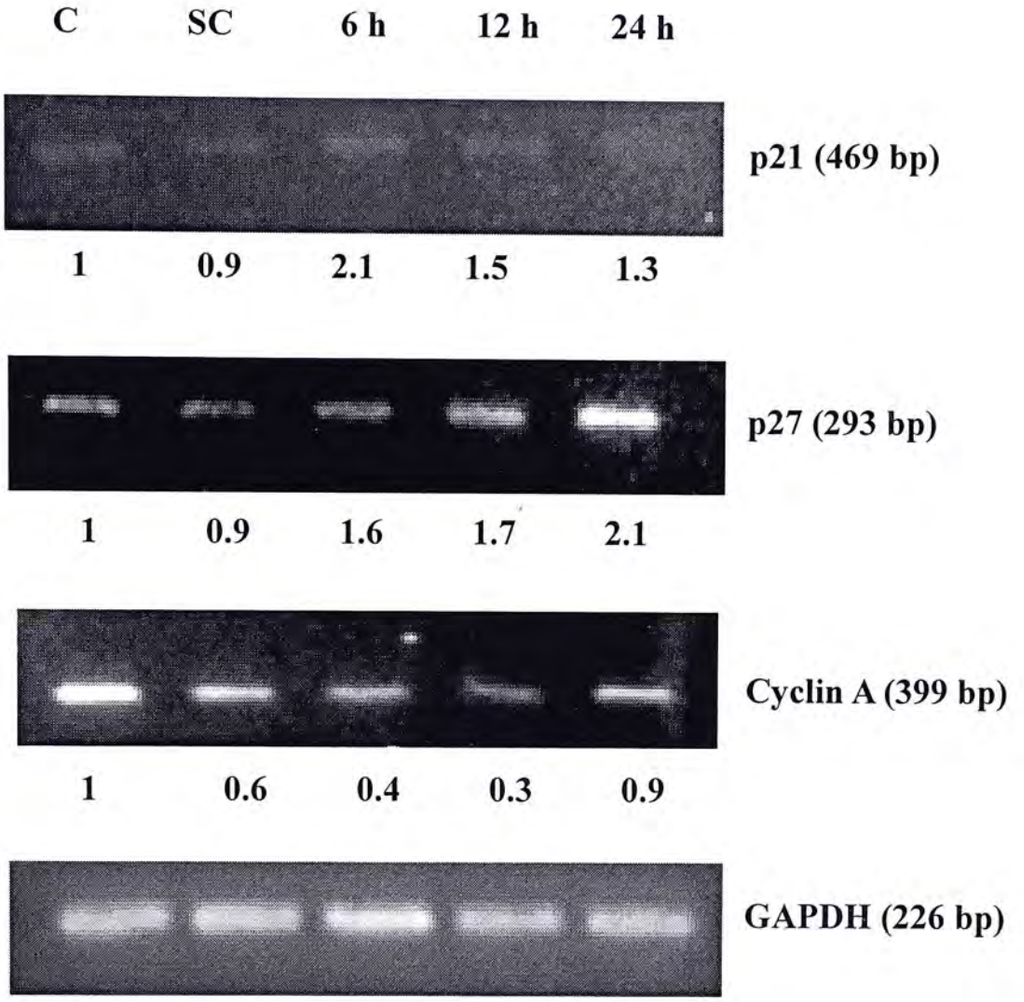



**Fig. 3.15: Analysis of the cell cycle-related gene expression in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by RT-PCR.** WEHI-3B JCS cells ($10^6$ cells) were incubated with 150 μM CLA-mix at 37°C for different time intervals (6, 12 and 24 hours) or treated with medium (C) or ethanol (SC) as controls. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding medium control.

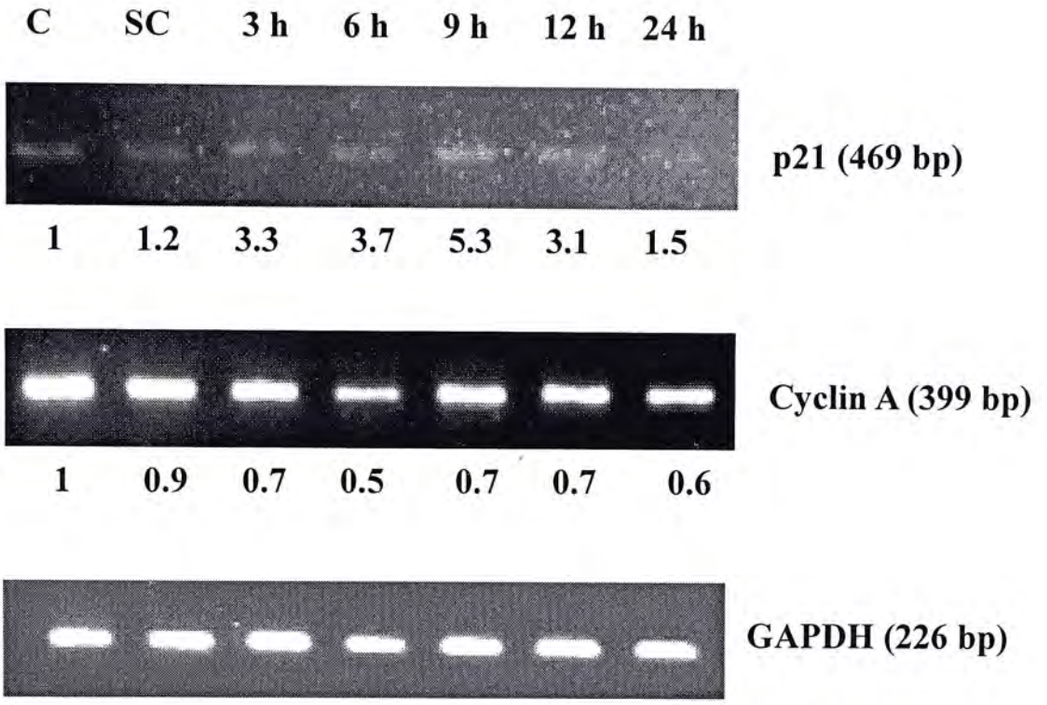



**Fig. 3.16: Analysis of the cell cycle-related gene expression in the 9E, 11E-CLA-treated myelomonocytic leukemia WEHI-3B JCS cells by RT-PCR.** WEHI-3B JCS cells ($10^6$ cells) were incubated with 150 μM 9E, 11E-CLA at 37°C for different time intervals (3, 6, 9, 12, and 24 hours) or treated with medium (C) or ethanol (SC) as controls. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding medium control.

### 3.2.7 Effect of CLA-mix and its isomer on the *In Vivo* Tumorigenicity of the WEHI-3B JCS Cells

So far, CLA-mix and the 9E, 11E-CLA isomer were found to inhibit the tumor cell growth of the murine myelomonocytic leukemia WEHI-3B JCS cells *in vitro,* and to induce $G_0/G_1$ phase arrest in the cell cycle of WEHI-3B JCS cells by modulating the expression of certain cell cycle-regulatory genes. It was, therefore, of interest to know whether they could also demonstrate the growth-inhibitory activity on tumor cells *in vivo*. WEHI-3B JCS cells were incubated for 8 hours at 37°C with two different concentrations of CLA-mix and the 9E, 11E-CLA isomer. The cells were washed with RPMI medium and injected i.p. into BALB/c mice, in groups of five, at $3 \times 10^6$ cells per mouse. Leukemia cells were harvested from the peritoneal cavity of mice 12 days following tumor inoculation. As shown in Figures 3.17 and 3.18, pre-treatment of WEHI-3B JCS cells with either CLA-mix or the 9E, 11E-CLA isomer could significantly reduce the tumor cell growth of WEHI-3B JCS cells in syngeneic mice in a dose-dependent manner.

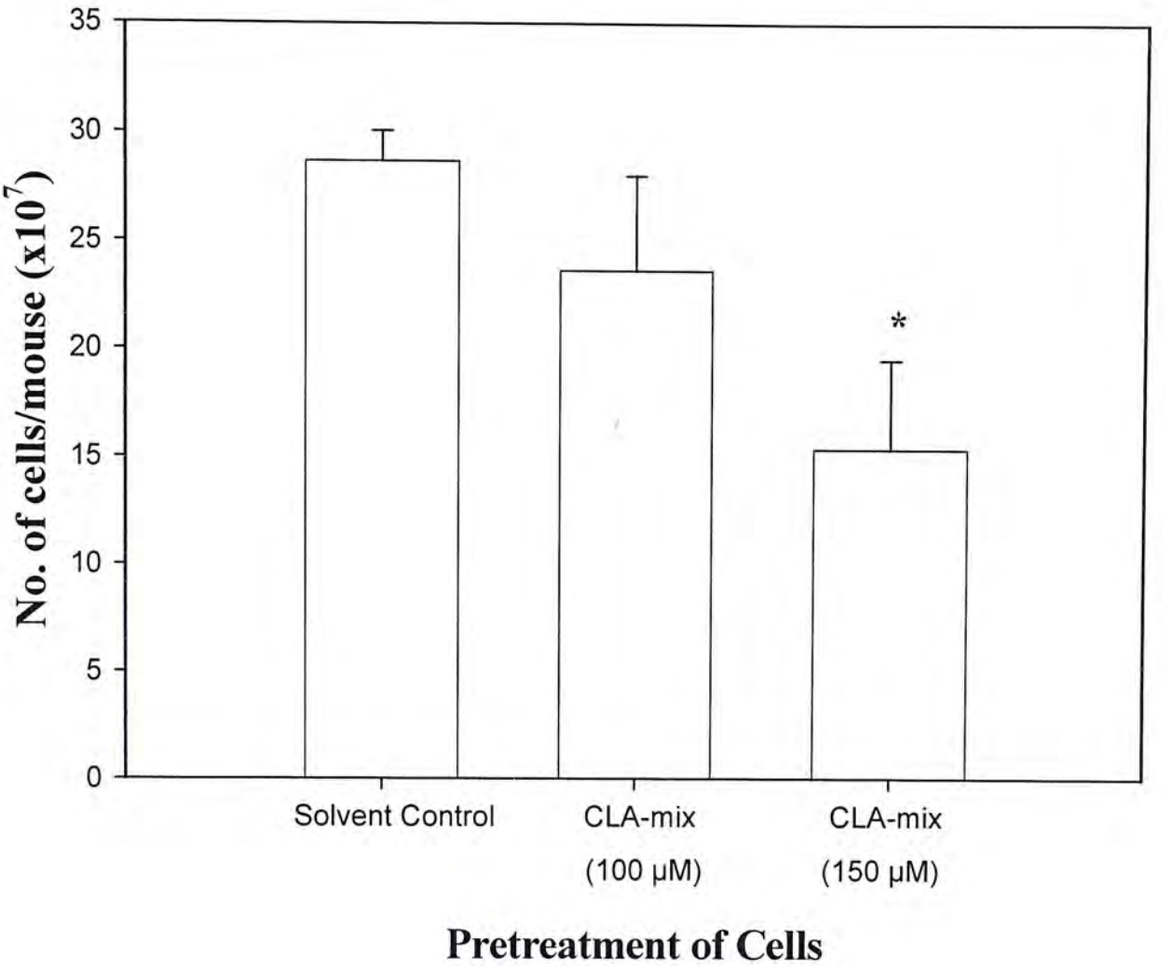



**Fig. 3.17: Effect of *in vitro* pre-treatment of myelomonocytic leukemia WEHI-3B JCS cells with CLA-mix on their tumorigenicity in syngeneic BALB/c mice.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with solvent control or with two different concentrations (100 µM and 150 µM) of CLA-mix for 8 hours at 37°C. The cells were then washed thrice with plain RPMI medium. Viable WEHI-3B JCS cells ($3 \times 10^6$) were injected i.p. into each BALB/c mouse in groups of five. Leukemia cells recoverable from the peritoneal cavity of mice were counted at day 12 post-tumor inoculation and results were expressed as percentage of inhibition of leukemic cell growth *in vivo*. Significantly different from untreated cells: * $p < 0.01$.

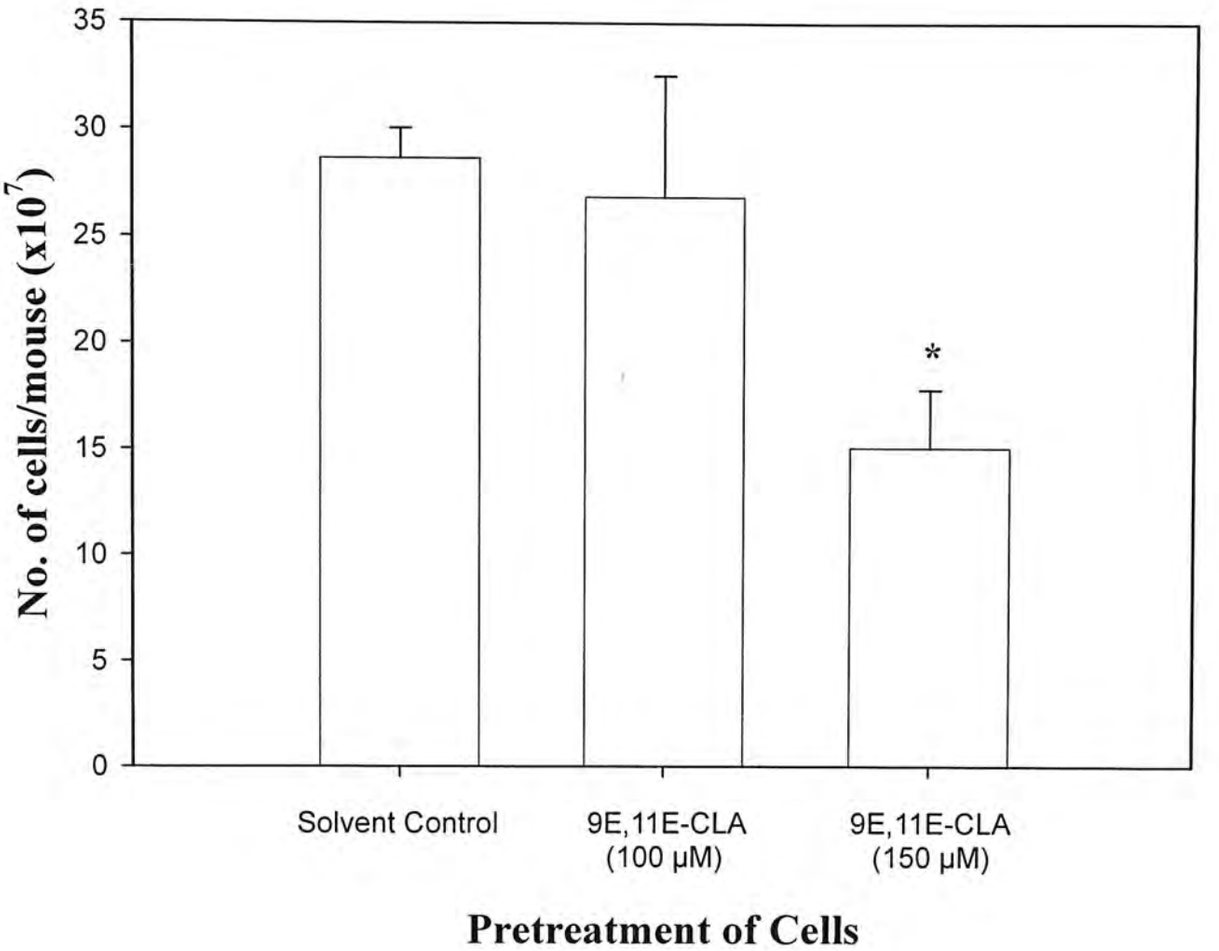



**Fig. 3.18: Effect of *in vitro* pre-treatment of murine myelomonocytic leukemia WEHI-3B JCS cells with 9E, 11E-CLA on their tumorigenicity in syngeneic BALB/c mice.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with solvent control or with two different concentrations (100 µM and 150 µM) of 9E, 11E-CLA for 8 hours at 37°C. The cells were then washed thrice with plain RPMI medium. Viable WEHI-3B JCS cells ($3x10^6$) were injected i.p. into each BALB/c mouse in groups of five. Leukemia cells recoverable from the peritoneal cavity of mice were counted at day 12 post-tumor inoculation and results were expressed as percentage of inhibition of leukemic cell growth *in vivo*. Significantly different from untreated cells: * $p < 0.001$.

## 3.3 Discussion

In this chapter, the effects of CLA-mix on the proliferation of murine myelomonocytic leukemia WEHI-3B JCS cells were examined. It was found that the growth of WEHI-3B JCS cells was inhibited dose-dependently by CLA-mix, with an estimated $IC_{50}$ value of approximately 150 μM following 48 hours of incubation. Similar growth-inhibitory effect of CLA-mix could also be demonstrated in various murine and human leukemia cell lines, including M1, HL-60, NB4, and K-562, as well as in the human lymphoma U-937 cells. Moreover, when compared to its parental fatty acid, linoleic acid (LA), and its four isomers, CLA-mix exhibited the most potent inhibition on the proliferation of WEHI-3B JCS cells. At 200 μM of fatty acid, CLA-mix showed a 62% growth inhibition on WEHI-3B JCS cells whereas LA showed only 22% of growth inhibition. Our results are in line with an earlier report showing that at similar concentrations, the cytostatic and cytotoxic effects of CLA were more pronounced than LA (Shultz et al., 1992). In fact, LA has been regarded as a promoter of carcinogenesis (Kilian *et al.*, 2003) and is known to induce tumor formation (Maggiora *et al.*, 2004). The differential effect of LA and CLA on the proliferation of tumor cells had also been reported previously. For example, Cunningham *et al.* (1997) showed that LA stimulated the growth of human breast cancer MCF-7 cells while CLA was inhibitory. On the other hand, Kim *et al.* (2002) demonstrated that CLA markedly inhibited the growth of human colon cancer Caco-2 cells, in contrast, LA slightly increased Caco-2 cell growth. Moreover, LA significantly reduced Fas-mediated apoptosis in human colorectal carcinoma CX-1 and CCL-188 cells, suggesting that such an inhibitory effect might protect the tumor

cells from lymphocyte-mediated apoptosis signaling through the Fas receptor (Meterissian *et al.*, 2000). In the present study, among the four CLA isomers tested, we found that 9E, 11E-CLA, 10E, 12Z-CLA and 9Z, 11E-CLA had similar anti-proliferation activity while the 9Z, 11Z-CLA isomer was less potent. It is known that 9Z, 11E-CLA and 10E, 12Z-CLA are two predominant isomers found in the commercial preparations of CLA mixtures. The individual potencies of these 2 CLA isomers on the proliferation of tumor cells had been investigated. It had shown that 10E, 12Z-CLA but not 9Z, 11E-CLA exhibited strong cytotoxic effect on the rat hepatoma dRLh-84 cells *in vitro* (Yamasaki *et al.*, 2002). Similarly, Kim *et al.* (2002b) showed that 10E, 12Z-CLA inhibited the growth of human colon cancer Caco-2 cells dose-dependently in serum-free medium whereas 9Z, 11E-CLA had no effect. On the other hand, Palombo *et al.* (2002) demonstrated that the 10E, 12Z-CLA exhibited the greatest inhibitory effect on the proliferation of two human colorectal cancer cell lines (HT-29 and MIP-101) whereas both 10E, 12Z-CLA and 9Z, 11E-CLA isomers were moderately effective against the human prostate cancer cell line PC-3. In contrast, a recent report showed that the 10E, 12Z-CLA was more effective than 9Z, 11E-CLA isomer in inhibiting PC-3 cell proliferation, since 10E, 12Z-CLA isomer seems to exert its effect through modulation of the cell apoptosis and cell cycle control, whereas the 9Z, 11E-CLA isomer affects arachidonic acid metabolism (Ochoa *et al.*, 2004). Interestingly, it had been reported that the 10E, 12Z-CLA isomer could inhibit the growth-factor-induced proliferation of the human breast cancer MCF-7 cells whereas the 9Z, 11E-CLA isomer had no effect. However, when the MCF-7 cells were cultured in medium supplemented with 1% FBS, 9Z, 11E-CLA was found to be more potent than 10E, 12Z-CLA in inhibiting MCF-7 cell proliferation, suggesting that these two CLA isomers have separate mechanisms and different targets of actions

(Chujo *et al.*, 2003). In contrast to these findings, our results showed that both the 10E, 12Z-CLA isomer and the 9Z, 11E-CLA isomer were equally effective in inhibiting the proliferation of the myeloid leukemia WEHI-3B JCS cells, though 10E, 12Z-CLA was a better inducer of $G_0/G_1$ cell cycle arrest than 9Z, 11E-CLA in WEHI-3B JCS cells. In addition, the 9Z, 11Z-CLA was found to be the least potent growth-inhibitor among all the isomers examined. Our results are also at variance with a recent report showing that 9Z, 11Z-CLA and 9Z, 11E-CLA being the most potent and least potent isomers respectively in the growth inhibition of the MCF-7 cells (Tanmahasamut *et al.*, 2004). Taken together, these findings suggest that the anti-proliferative activity of CLA isomers depends not only on the culture conditions but also on the type of tumor targets.

In one study using the human breast cancer MCF-7 cells, it was found that there was no synergistic or opposing action between CLA isomers as CLA-mix, which contains almost the same amount of the predominant isomers, 9Z, 11E-CLA and 10E, 12Z-CLA, showed an intermediate anti-proliferative activity between these two isomers (Chujo *et al*, 2003). By contrast, our results showed that interactions among individual CLA isomers might exist as CLA-mix suppressed the growth of WEHI-3B JCS cells to a greater extent than each of its four isomers.

In order to determine whether the observed anti-proliferative activity of CLA-mix was owing to its cytostatic or cytotoxic effects, the trypan blue exclusion assay was carried out to test for its cytotoxicity. Our results showed that CLA-mix, at concentrations ranged from 25 to 200 μM, exhibited no cytotoxicity on the WEHI-3B JCS cells following incubation for 24, 48, and 72 hours. Additionally, we also demonstrated that the growth-inhibitory effect of CLA-mix was not due to the solvent

effect, since the highest concentration of ethanol (0.1%) used in our study exhibited little, if any, growth-inhibitory effect on the WEHI-3B JCS cells after 48 hours of incubation. Moreover, our results also indicated that CLA-mix (25-200 μM) exhibited minimal, if any, cytotoxic effect on normal myeloid cells such as the thioglycollate-elicited murine peritoneal macrophages after 48 hours of incubation.

Kinetic study of the anti-proliferative activity of CLA-mix on WEHI-3B JCS cells showed that CLA-mix could inhibit their proliferation in a time-dependent manner. Interestingly, such growth-inhibitory effect of CLA-mix was partially reversible when the WEHI-3B JCS cells were exposed to CLA-mix for a short incubation period (6 and 12 hours). These results suggest that CLA-mix might exert its anti-proliferative effect on WEHI-3B JCS cells within the first 24 hours and that longer incubation time will result in irreversible changes.

Modulation on the deregulated cell cycle of tumor cells could, very often, establish novel therapeutic approaches for a variety of cancers. In fact, many conventional chemotherapeutic agents intervene at multiple phases in the cell cycle (Johnson and Walker, 1999). DNA damaging drugs like cisplatin, nitrogen mustard, cyclophosphamide, and chlorambucil can cause cell cycle arrest at both the $G_1$/S and the $G_2$/M checkpoints. Moreover, nucleoside analogs such as hydroxyurea, gemcitibine, and difluorodeoxyuridine can also activate the $G_1$ checkpoint arrest. In this study, our results showed that 24-hour treatment with CLA-mix increased the percentage of WEHI-3B JCS cells in $G_0/G_1$ phase in a dose-dependent manner which was accompanied by a corresponding reduction in the percentages of cells in the S phase. Among the four CLA isomers being investigated, the 10E, 12Z-CLA increased the percentage of WEHI-3B JCS cells in $G_0/G_1$ phase to the greatest extent. In fact,

our results are in line with the recent findings of Kemp *et al.* (2003) who had reported that the 10E, 12Z-CLA isomer is more effective than the 9Z, 11E-CLA isomer in inducing cell cycle arrest at the $G_1$ phase of the human breast cancer MCF-7 cells. Nevertheless, our results suggest that not all of the CLA isomers can modulate the cell cycle of WEHI-3B JCS cells as the 9Z, 11Z-CLA isomer did not exert any effect on the cell cycle.

As depicted in Fig 3.19, it has been well established that cell cycle progression is primarily regulated by the sequential activation and inactivation of certain cyclin-dependent kinases (cdks) through the periodic synthesis and destruction of cyclins (Eastman A, 2004; Johnson and Walker, 1999). The $G_1$/S-promoting cyclin/cdk complexes refer to cyclin D1/2/3/cdk4/6, cyclin E1/2/cdk2, and cyclin A/cdk1/2 (Kastan and Bartek, 2004), which are regulated by a stoichiometric combination of cyclin-dependent kinase inhibitors (CKIs) such as $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ of the Cip/Kip Family (Senderowicz, 2004; Johnson and Walker, 1999). In fact, ectopic expression or amplification of cyclin D, E, and A has been reported in a variety of cancers (Johnson and Walker, 1999). In order to halt cell cycle progression through $G_1$ phase, $p21^{CIP1/WAF1}$ is capable of inhibiting the cyclin D-, E-, and A-dependent kinase activities (el-Deiry *et al.*, 1993), while $p27^{KIP1}$ is responsible to silence the cyclin E- and A-dependent kinase activities of cdk2 (Massague, 2004).

Many mutated oncogenes and tumor suppressor genes are also associated with faulty $G_1$ control. Among all, *p53* is the most frequently mutated tumor suppressor gene in human cancer (Sherr, 2004). The p53 protein, regarded as the guardian of the genome, plays a pivotal role in regulating the cell cycle at the $G_1$/S interval or triggering apoptosis in response to DNA damage (Kemp *et al.*, 2003; Eastman, 2004).

p53 regulates the cell cycle, at least in part, by inducing the transcription of $p21^{CIP1/WAF1}$. In the present study, the expression of several cell cycle-regulatory genes was examined. The mRNA levels of the *p53* gene were increased following exposure to CLA-mix and 9E, 11E-CLA. Moreover, both CLA-mix and 9E, 11E-CLA increased the expression of the $p21^{CIP1/WAF1}$ gene but decreased the expression of the *cyclin A* gene. In addition to the CKI $p21^{CIP1/WAF1}$, CLA-mix also increased the mRNA level of another CKI, the $p27^{KIP1}$, which has been known to inhibit cyclin/cdk complexes such as the cyclin A/cdk2 from promoting $G_1$ to S phase transition (Russo *et al.*, 1996). The hypothetical pathways by which CLA could activate cell cycle arrest at the $G_1$ phase were shown in Fig. 3.20. Indeed, our results are in agreement with many published work which showed that CLA can regulate tumor cell cycle by modifying the expression of cyclins, cdk inhibitors, and other checkpoint proteins (Belury, 2002). Ip *et al.* (2001) had demonstrated that feeding CLA-mix for 4 weeks reduced the expression of cyclins D1 and A in the terminal end buds and alveolar clusters of rat mammary epithelium. More recently, the anti-proliferative activity of CLA-mix in human breast cancer MCF-7 cells and colon cancer HCT 116 cells has been attributed to the up-regulation of wild-type *p53* gene expression (Kemp *et al.*, 2003). In this study, CLA-mix elicited cell cycle arrest at the $G_1$ phase and induced the accumulation of CKIs including $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins. Besides, CLA-mix also reduced the expression of proteins required for $G_1$ to S-phase transition involving cyclins D1 and E (Kemp *et al.*, 2003). A more recent report has also shown that 24-hour treatment with CLA-mix induced $G_1$ arrest by over-expressing the $p21^{CIP1/WAF1}$ gene in human colon carcinoma HT-29, HCT116, and SW480 cell lines (Lim *et al.*, 2005). Moreover, in addition to the CLA-mix, the 10E, 12Z-CLA isomer has also been shown to decrease the proliferation of the human prostate carcinoma

PC-3 cells by over-expressing the $p21^{CIP1/WAF1}$ gene. By contrast, the 9Z, 11E-CLA isomer did not alter the mRNA levels of the $p21^{CIP1/WAF1}$ gene in the PC-3 cells (Ochoa *et al.*, 2004). To our knowledge, there are no previous reports showing that the 9E, 11E-CLA isomer could arrest tumor cell cycle, and we are the first to show that this CLA isomer could trigger $G_1$ arrest by modulating cell cycle-regulatory genes including *p53*, $p21^{CIP1/WAF1}$, and *cyclin A*.

Although CLA has been found to induce $p21^{CIP1/WAF1}$ gene expression, however, whether CLA-induced $G_1$ arrest is p53-dependent remains controversial. Kemp *et al.* (2003) demonstrated that CLA did not cause alteration on the protein levels of p53, $p21^{CIP1/WAF1}$, p27, and cyclin E in the *p53*-mutant MCF-7 cells and *p53*-deficient human colon cancer HCT116 cells, suggesting that CLA-induced $G_1$ arrest depends on the action of the wild-type *p53* gene. However, Lim *et al.* (2005) suggested that wild type p53 is not essential for induction of $p21^{CIP1/WAF1}$ by CLA, since CLA addition also led to increased p21 expression in both the human colon cancer HT-29 and SW480 cells with the mutant *p53* gene. In addition to the increased protein expression of $p21^{CIP1/WAF1}$ in the HT-29 cells, CLA-induced $G_1$ arrest was accompanied by the reduced expressions of other cell cycle-regulatory proteins such as cyclin D1, cyclin E, cyclin A, phosphorylated Rb protein, and proliferating cell nuclear antigen (PCNA), and with the reduced kinase activities of cdk2 and cdk4 (Lim *et al.*, 2005). Nevertheless, more extensive studies should be carried out in other tumor cell lines so as to determine whether the wild-type *p53* gene plays a pivotal role in regulating the $G_1$ checkpoint control following exposure to CLA.

Since CLA was found to exhibit anti-proliferative activity and triggered $G_1$ phase arrest in the leukemia WEHI-3B JCS cells, therefore, the suppressive effect of CLA

on the tumorigenicity of the WEHI-3B JCS cells in syngeneic BALB/c mice was also examined. The results showed that pretreatment of WEHI-3B JCS cells *in vitro* with either the CLA-mix or the 9E, 11E-CLA isomer could decrease the *in vivo* growth of the leukemia cells in syngeneic mice. Interestingly, Visonneau *et al.* (1997) had reported that 1% dietary CLA could block the local growth and systemic spread of human breast adenocarcinoma cells in SCID mice, indicating CLA may exert its anti-tumor effect independent of the host immune system. Nevertheless, the mechanisms by which CLA can mediate its anti-tumor effect *in vivo* await further investigations.

In conclusion, in this chapter it was demonstrated that CLA-mix and some of its isomers could exhibit growth-inhibitory effect on the myeloid leukemia cells. Activation of cell cycle arrest could be one of the possible mechanisms contributing to the observed anti-proliferative activity of CLA. Other possible mechanisms including the induction of apoptosis and differentiation of myeloid leukemia cells by CLA would be examined and discussed in detail in chapters 4 and 5 respectively.

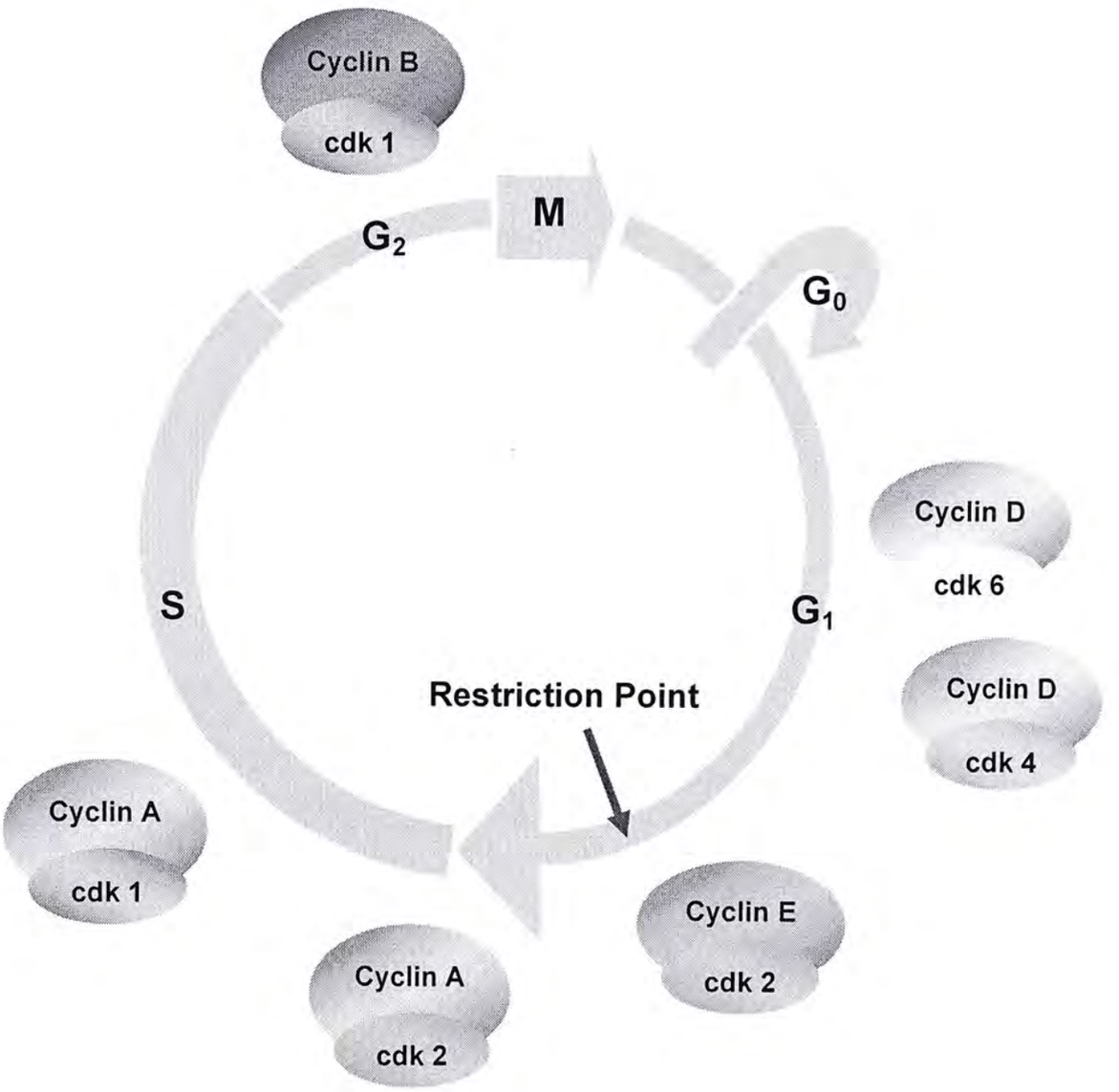



**Fig. 3.19: Regulation of cell cycle progression by cyclin-dependent kinases (cdks) and cyclins.** Cell cycle progression is the result of enzymatic phosphorylation of various cell cycle proteins by the serine/threonine kinases, also known as cdks. These cell cycle regulators periodically form complexes with proteins called cyclins. The main cyclin/cdk complexes responsible for $G_1$/S are cyclin D/cdk4/6, cyclin E/cdk2, and cyclin A/cdk1/2. Cyclin A/cdk1/2 complexes are in charge of S phase entry and transition, whereas cyclin B/cdk1 complex is the main cyclin/cdk complex responsible for $G_2$ exit and M phase entry.

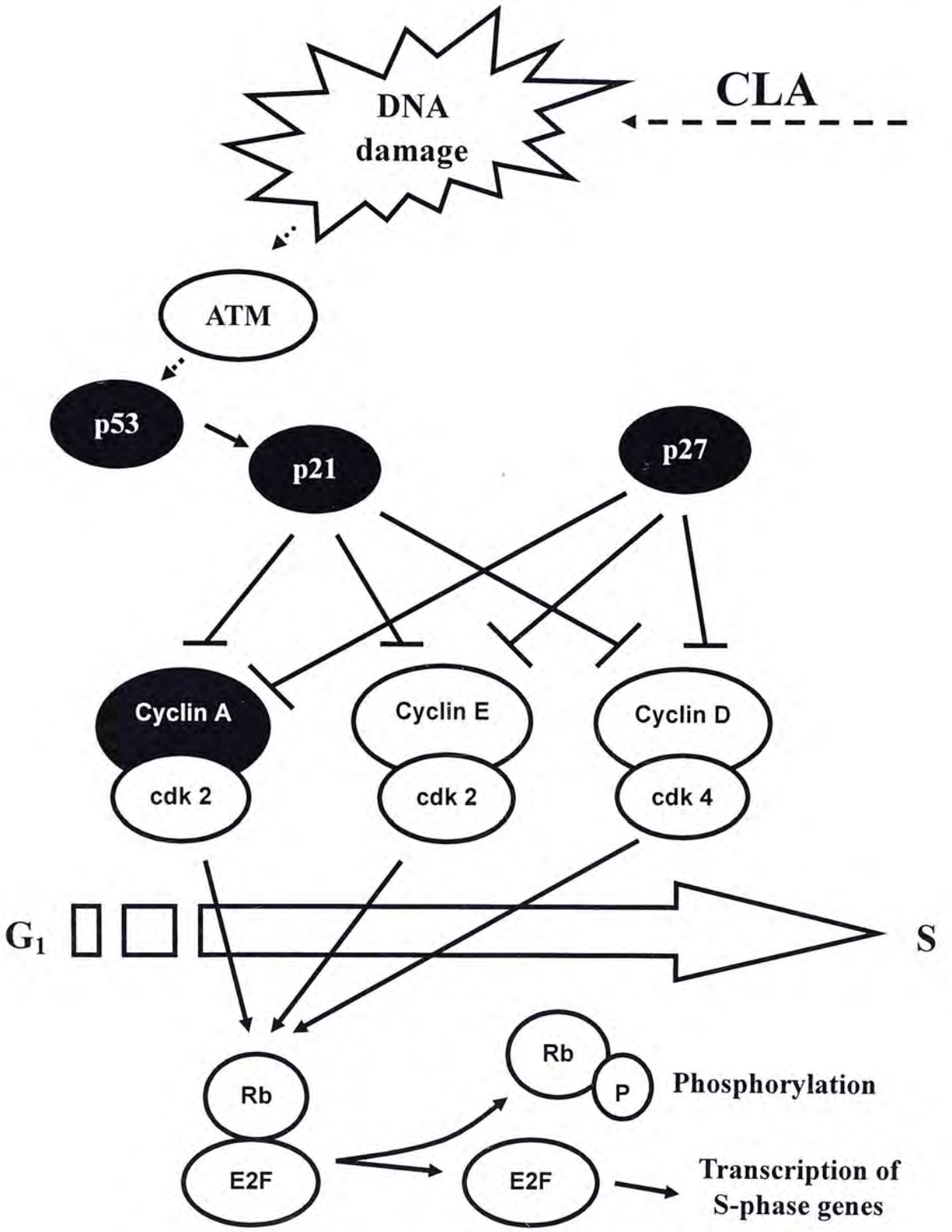



**Fig. 3.20: Schematic representation of $G_1$/S transition.** CLA may promote progression to S phase through phosphorylation of the retinoblastoma protein (Rb) by cyclin/cdk complexes, which are in turn regulated by cdk inhibitors such as p21 and p27. DNA synthesis (S phase) occurs when Rb is phosphorylated, releasing the transcription factor E2F. $G_1$ phase arrest following DNA damage is owing to the transcriptional activation of p53, leading to ectopic expression of p21 and thereby inhibition of the $G_1$/S-promoting cyclin/cdk complexes.
(Modified from Senderowicz, 2004)

# CHAPTER 4

# STUDIES ON THE APOPTOSIS-INDUCING ACTIVITY OF CONJUGATED LINOLEIC ACID ON MYELOID LEUKEMIA CELLS

# 4.1 Introduction

Cell death is a vital cellular process for animal development. There are three modes of cell death that are currently understood as autophagy, necrosis, and apoptosis (Crow *et al.*, 2004). Autophagy is exemplified by the degradation of proteins and organelles in the lysosomal pathway through which their constituents can be reused by the cell (Klionsky and Emr, 2000). On the other hand, necrosis and apoptosis can be distinguished by distinct morphological and molecular features of the dying cells (Strasser *et al.*, 2000). The morphological changes that occur during apoptosis include chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing, and nuclear membrane breakdown; whereas the molecular changes that arise during apoptosis involve internucleosomal DNA cleavage and randomization of phosphatidyl serine (PS) distribution between the inner and outer layers of the plasma membrane. Moreover, there are also apoptotic bodies formed during apoptosis (Jiang and Wang, 2004), which are rapidly engulfed by phagocytes such as macrophages and neutrophils after apoptosis.

As a matter of fact, apoptosis is an evolutionarily preserved process that plays pivotal roles in embryonic development and in the homeostasis, remodeling, surveillance, and host defenses of postnatal tissues (Crow *et al.*, 2004). Very often, the loss of apoptosis is the hallmark of human malignancies. Therefore, reactivating the apoptotic machinery in tumor cells appears to be an attractive approach to cancer treatment. Many chemotherapeutic drugs and γ-irradiation induce apoptosis in tumor cells (Strasser *et al.*, 2000). For instance, anti-tumor drugs such as etoposide and mitoxanthrone (Kaufmann, 1989; Bhalla *et al.*, 1995) with strong pro-apoptotic

activity but minimal cytotoxicity would be expected to offer potential chemotherapeutic effects on myeloid leukemia cells.

Apoptosis is mediated by two central pathways, namely the "intrinsic" and "extrinsic" pathways (Senderowicz, 2004). The "intrinsic" pathway is employed to eliminate cells in response to chemotherapeutic drugs, DNA damage, ionizing radiation, and also oxidative stress (Boatright and Salvesen, 2003; Crow *et al.*, 2004). On the other hand, the "extrinsic" pathway is responsible for eliminating the unwanted cells during development, educating our immune system, and triggering the immune-system-mediated tumor removal (immunosurveillance) (Boatright and Salvesen, 2003). The "intrinsic" pathway is characterized by dissipation of mitochondrial membrane potential ($\Delta \phi$m), release of mitochondrial cytochrome c (cyt c) leading to subsequent activation of caspase-9; whereas the "extrinsic" pathway is characterized by the activation of death receptors such as Fas and the resultant activation of caspase-8. In fact, the two signaling cascades may be sometimes entwined. For example, caspase-9 and caspsae-8 from both pathways can cleave and activate effector procaspase-3 into activated caspase-3 (Nagata, 1999; Bratton *et al.*, 2001). Activated caspase-3 then cleaves specific substrates including poly (ADP-ribose) polymerase (PARP), resulting in morphological and biochemical changes of apoptosis (Li *et al.*, 1997; Hengartner, 2000).

In response to death signals, the decision of a cell to undergo apoptosis may depend upon the complex interplay between the "intrinsic" and "extrinsic" signaling cascades. In addition, a great deal of evidence has indicated that the intracellular redox status is also one of the key mediators of apoptosis in many cell systems. Mitochondria are a major source of reactive oxygen species (ROS) (Droge, 2002). It has been previously reported that ROS may trigger cyt c release by promoting

mitochondrial permeability transition (MPT) through oxidation of thiol groups on the adenine nucleotide translocator (ANT) of the inner mitochondrial membrane (IMM), suggesting that mitochondria are targets of ROS in apoptosis (Kanno *et al.*, 2004). Moreover, the novel coupling between ROS and Fas aggregation appears to play a significant role in apoptosis induced by DNA-damaging agents in the Fas-expressing leukemia cells (Huang *et al.*, 2003). Therefore, ROS can also trigger apoptosis by Fas-dependent pathway.

In this chapter, the apoptosis-inducing activity of the CLA-mix and four CLA isomers, namely 9Z, 11E-CLA, 10E, 12Z-CLA, 9Z, 11Z-CLA, and 9E, 11E-CLA, on the murine myelomonocytic leukemia WEHI-3B JCS cells was examined by using the DNA fragmentation assay and quantified by using the ELISA kit. One of the CLA isomers which showed the greatest potency to induce apoptosis of the WEHI-3B JCS cells was used for further mechanistic studies. In order to reveal which apoptotic pathway(s) has/have been triggered by CLA-mix or its isomer, the changes in the mitochondrial membrane potential were studied by the JC-1 staining method, and the activities of certain caspases such as caspases-3, -8, and -9 were measured using specific fluorometric substrates. The effects of CLA-mix- and CLA isomer in inducing the transcriptional and translational activation of the apoptosis-regulatory genes in the WEHI-3B JCS cells were elucidated by RT-PCR and Western blot analysis, respectively. Furthermore, the possible association of CLA-induced apoptosis and the increased production of reactive oxygen species (ROS) by WEHI-3B JCS cells was analyzed by flow cytometry. Finally, the effects of free radical scavengers, including N-acetylcysteine (NAC) and superoxide dismutase (SOD), on the CLA-induced apoptosis would be determined by the DNA fragmentation assay.

# 4.2 Results

## 4.2.1 Induction of Apoptosis in Both Murine and Human Myeloid Leukemia Cells by CLA

During apoptosis, several morphological features can be observed and of which, DNA fragmentation is often used as a positive indicator for the occurrence of apoptosis. In the present study, whether CLA-mix can induce apoptosis in the murine myelomonocytic leukemia WEHI-3B JCS cells and human promyelocytic leukemia HL-60 cells was determined by the DNA fragmentation assay. As shown in Fig. 4.1 and 4.2, CLA-mix triggered DNA fragmentation in both WEHI-3B JCS cells and HL-60 cells dose-dependently. At its $IC_{50}$, (150 μM), CLA-mix caused DNA fragmentation in WEHI-3B JCS cells after 48 hours of incubation and a time-dependent response can also be demonstrated (Fig. 4.3). In addition to CLA-mix, several CLA isomers were also investigated for their apoptosis-inducing activity. As shown in Fig. 4.4, DNA fragmentation appeared in the WEHI-3B JCS cells treated with 150 μM 10E, 12Z-CLA and 150 μM 9E, 11E-CLA after 48 hours of incubation, whereas DNA fragments were not detected in cells treated with the same concentration of 9Z, 11E-CLA and 9Z, 11Z-CLA. Interestingly, the 9E, 11E-CLA isomer elicited stronger pro-apoptotic activity when compared to the 10E, 12Z-CLA isomer, which was known to induce apoptosis in a number of tumor cell types (Yamasaki *et al.*, 2002; Ochoa et al., 2004). The apoptosis-inducing activity of CLA-mix and its isomers was further confirmed by the Cell Death Detection ELISA$^{PLUS}$ kit (Fig. 4.5). This kit is used for quantifying the degree of apoptosis (Bourre *et al.*, 2002) based on the principle of quantitative sandwich-enzyme-immunoassay using mouse monoclonal antibodies to specifically

quantify the amount of mono- and oligonucleosomes present in the cytoplasmic fraction of cell lysates.

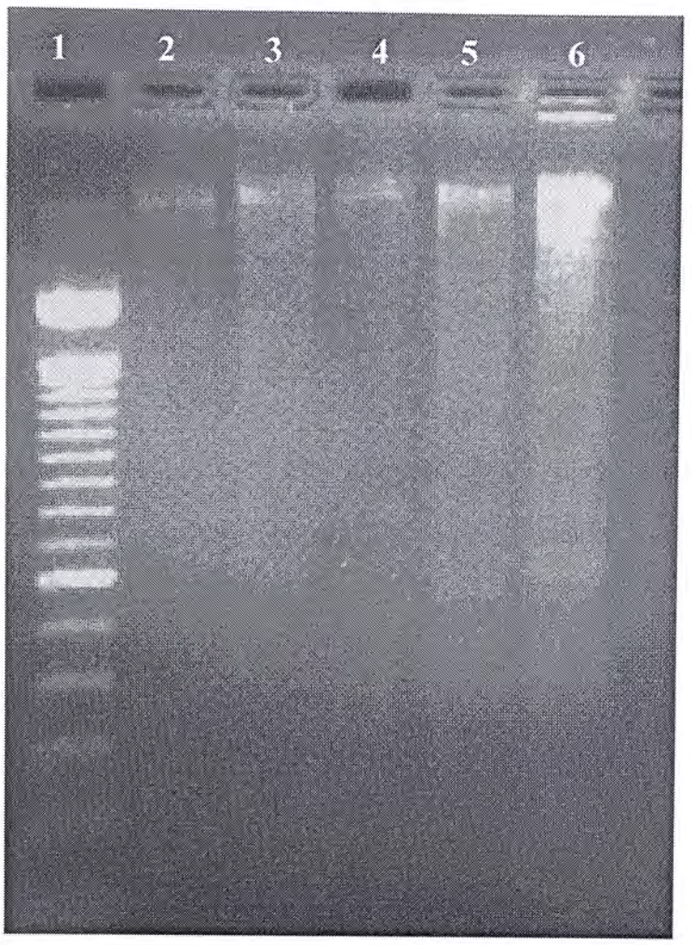



**Fig. 4.1: Dose response study for the induction of DNA fragmentation in CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated (Lane 2) or incubated with solvent control (0.1% ethanol) (Lane 3) or cultured with different concentrations of CLA-mix (Lane 4-6) at 37°C for 48 hours. Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA Markers

Lane 2: Untreated JCS cells

Lane 3: Solvent control-treated JCS cells

Lane 4: JCS cells treated with CLA-mix (50 μM)

Lane 5: JCS cells treated with CLA-mix (100 μM)

Lane 6: JCS cells treated with CLA-mix (150 μM)


**Fig. 4.2: Dose response study for the induction of DNA fragmentation in CLA-mix-treated human promyelocytic leukemia HL-60 cells.** HL-60 cells ($5x10^4$ cells/ml) were either untreated (Lane 2) or incubated with solvent control (0.1% ethanol) (Lane 3) or cultured with different concentrations of CLA-mix (Lane 4-6) at 37°C for 48 hours. Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA Markers

Lane 2: Untreated HL-60 cells

Lane 3: Solvent control-treated HL-60 cells

Lane 4: HL-60 cells treated with CLA-mix (50 μM)

Lane 5: HL-60 cells treated with CLA-mix (100 μM)

Lane 6: HL-60 cells treated with CLA-mix (150 μM)

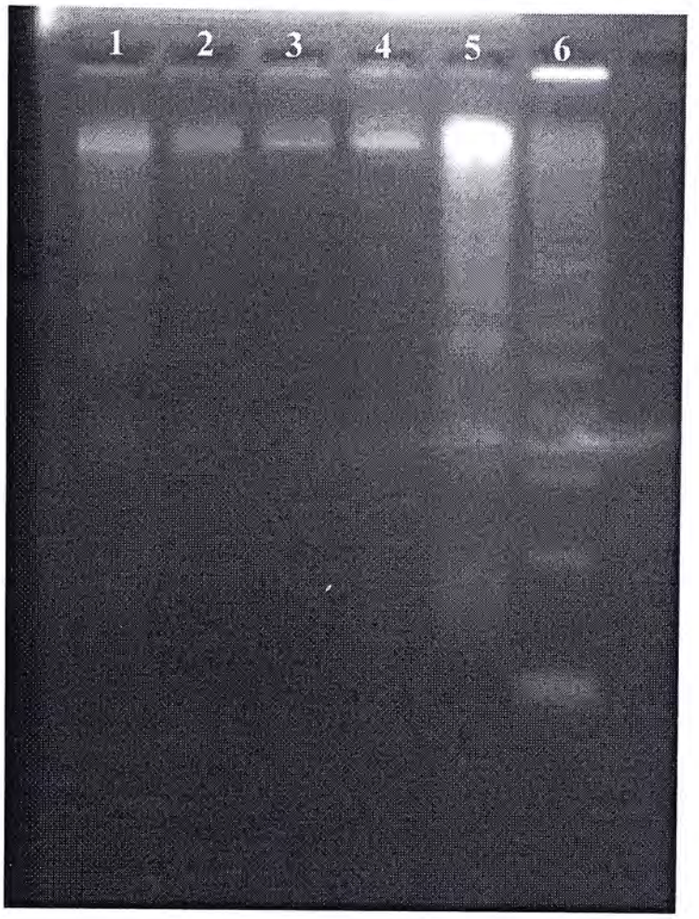



**Fig. 4.3: Kinetic study on the induction of DNA fragmentation in CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated (Lane 2) or incubated with solvent control (0.1% ethanol) (Lane 3) or cultured with 150 μM CLA-mix at 37°C for different periods of time (Lane 1, 4, 5). Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: JCS cells treated with CLA-mix for 48 hours

Lane 2: Untreated JCS cells

Lane 3: Solvent control-treated JCS cells

Lane 4: JCS cells treated with CLA-mix for 24 hours

Lane 5: JCS cells treated with CLA-mix for 72 hours

Lane 6: 100 bp DNA Markers

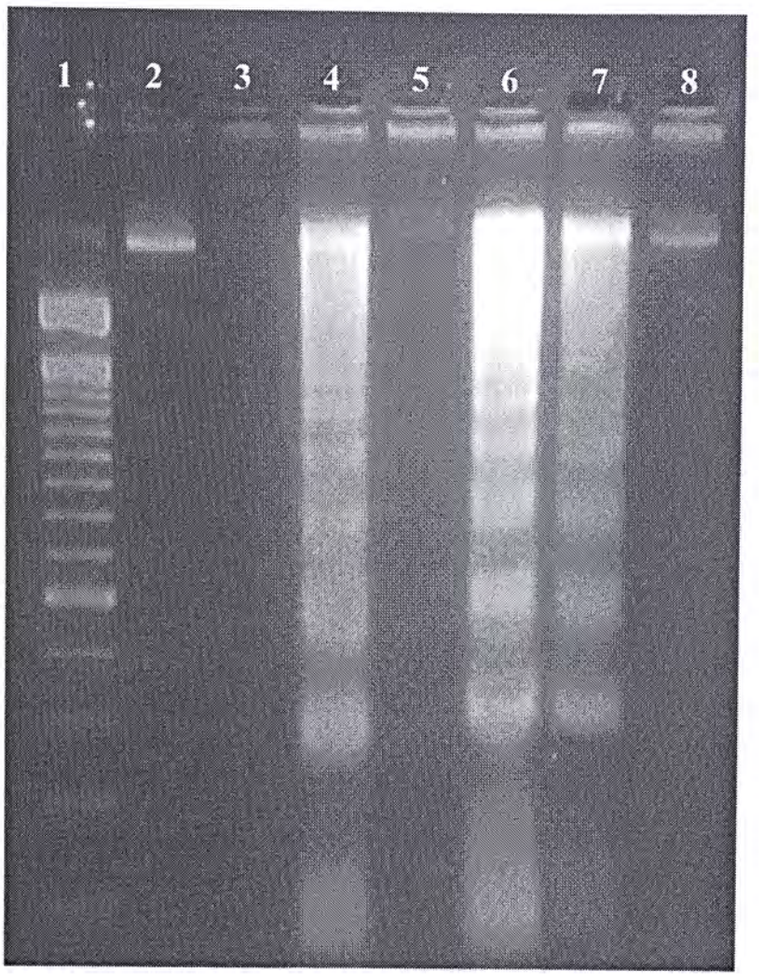



**Fig. 4.4: Induction of DNA fragmentation in murine myelomonocytic leukemia WEHI-3B cells following treatment with CLA-mix or its various isomers.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated (Lane 2) or incubated with solvent control (0.1% ethanol) (Lane 3) or cultured with 150 μM CLA-mix (Lane 4) and its various isomers (Lane 5-8) at 37°C for 48 hours. Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA Markers

Lane 2: Untreated JCS cells

Lane 3: Solvent control-treated JCS cells

Lane 4: JCS cells treated with CLA-mix (150 μM)

Lane 5: JCS cells treated with 9Z,11Z-CLA (150 μM)

Lane 6: JCS cells treated with 9E, 11E-CLA (150 μM)

Lane 7: JCS cells treated with 10E, 12Z-CLA (150 μM)

Lane 8: JCS cells treated with 9Z, 11E-CLA (150 μM)

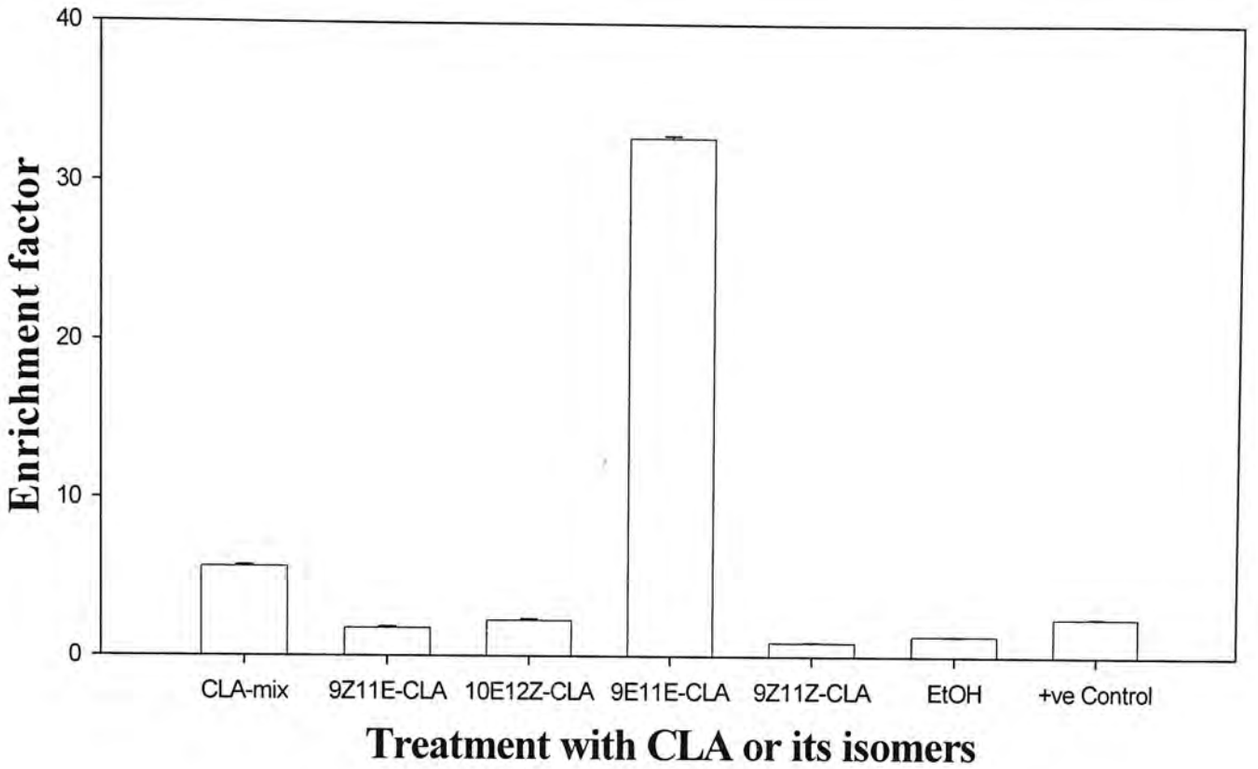



**Fig. 4.5: Induction of apoptosis in CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated or treated with 150 μM CLA-mix or its isomers at 37 °C for 48 hours. Apoptotic DNA fragments were detected using an ELISA kit. The degree of apoptosis was expressed as enrichment factor as described in detail in Chapter 2. The DNA-Histone-Complex, supplied by the manufacturer, is used as positive control in this study.

### 4.2.2 Effect of CLA and its Isomer on the Mitochondrial Membrane Potential of the WEHI-3B JCS Cells

Mitochondrial membrane depolarization is one of the putative mechanisms in the "intrinsic" apoptotic pathway leading to translocation of the apoptogenic proteins, such as cytochrome c (cyt c) and apoptosis-inducing factor (AIF), from mitochondria into cytosol, resulting in activation of caspase cascades. The mitochondrial membrane potential ($\Delta \phi m$) can be measured by the JC-1 dye, chemically known as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (Cossarizza *et al.*, 1993). JC-1 is a cationic dye that accumulates in mitochondria in a potential-dependent manner. It is used as an indicator to assess the $\Delta \phi m$ in cells by forming J-aggregates in the polarized mitochondrial membrane and emitting red fluorescence at 590 nm after excitation at 488 nm. By contrast, its monomeric form accumulates in depolarized mitochondrial membrane and emits green fluorescence at 525 nm (Dorrie *et al.*, 2001). The depolarization of mitochondrial membrane can be thereby detected as a reduced red/green fluorescence intensity ratio becasue the emission of fluorescence shifts from red to green (Smiley *et al.*, 1991; Di Lisa *et al.*, 1995). In the present study, WEHI-3B JCS cells were incubated with 150 μM CLA-mix or 150 μM 9E, 11E-CLA for 12, 24, 36, and 48 hours. The cells were then stained with the JC-1 dye and the red and green fluorescence were measured by flow cytometry. Our results showed that a time-dependent depolarization of $\Delta \phi m$ became significant after 36 hours of incubation with CLA-mix and 12 hours of incubation with 9E, 11E-CLA (Fig 4.6 and 4.7). About 54% and 77% cells had their mitochondrial membrane depolarized at 48 hours of treatment with CLA-mix and 9E, 11E-CLA, respectively.

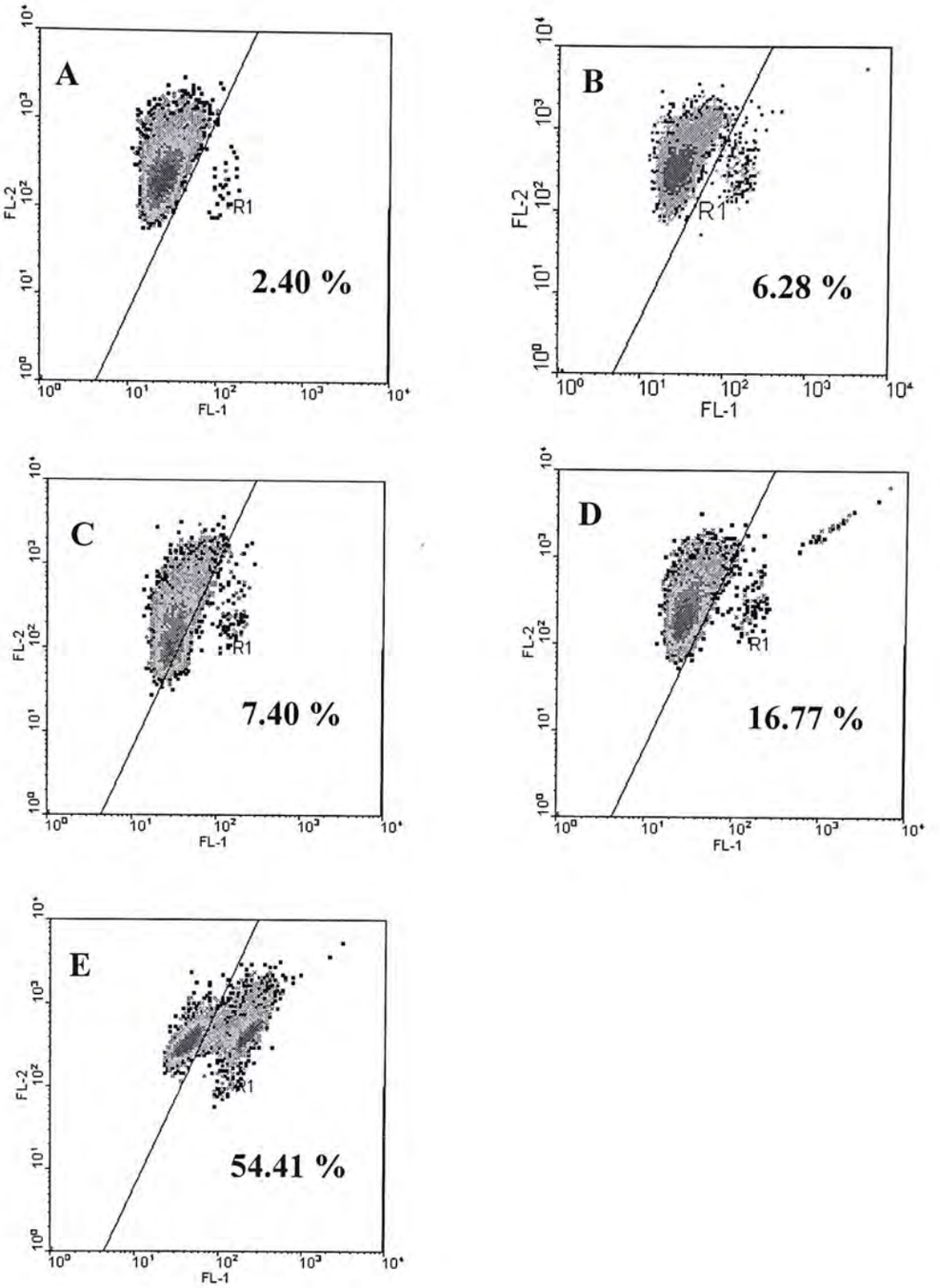



**Fig. 4.6: The effect of CLA-mix on the mitochondrial membrane depolarization in murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with (A) solvent control (0.1% ethanol), or 150 μM CLA-mix for (B) 12 hours, (C) 24 hours, (D) 36 hours, or (E) 48 hours and then stained with the mitochondria-selective JC-1 dye. Cells with normal polarized mitochondrial membranes at the top left quadrant emit red fluorescence. The number at the bottom right quadrant represented the percentage of cells that emit green fluorescence attributable to depolarized mitochondrial membranes.

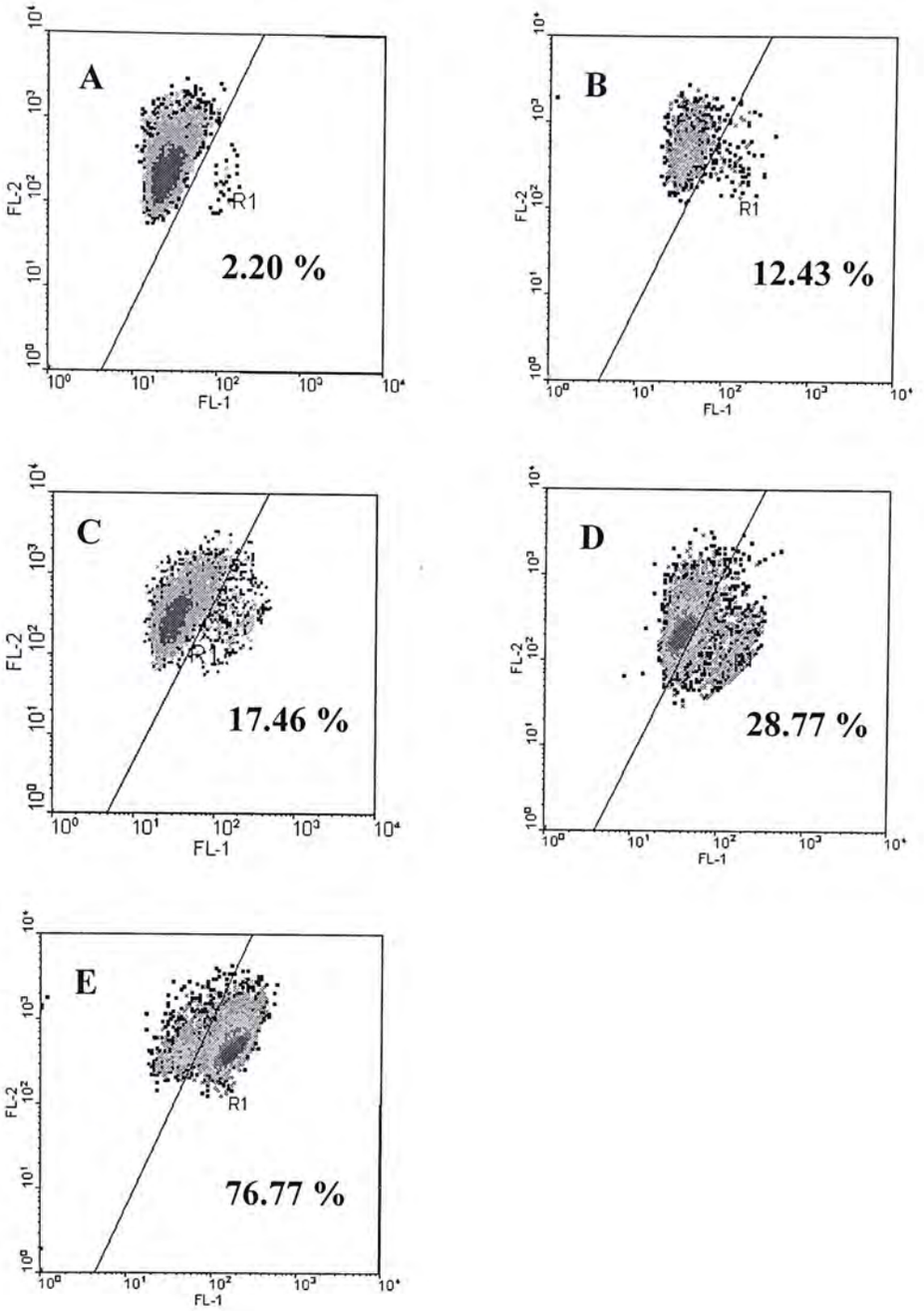



**Fig. 4.7: The effect of 9E, 11E-CLA on the mitochondrial membrane depolarization in myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with (A) solvent control (0.1% ethanol), or 150 μM 9E, 11E-CLA for (B) 12 hours, (C) 24 hours, (D) 36 hours, or (E) 48 hours and then stained with the mitochondria-selective JC-1 dye. Cells with normal polarized mitochondrial membranes at the top left quadrant emit red fluorescence. The number at the bottom right quadrant represented the percentage of cells that emit green fluorescence attributable to depolarized mitochondrial membranes.

### 4.2.3 Effect of CLA-mix and its Isomer on the Expression of Apoptosis-regulatory Genes of the Bcl-2 Family in the WEHI-3B JCS Cells

The Bcl-2 family of proteins can be divided into both anti-apoptotic and pro-apoptotic members. Mammalian anti-apoptotic Bcl-2 proteins which promote cell survival include Bcl-2 and Bcl-$_{XL}$ (Bcl-x protein long isoform). The Bcl-$_{XL}$ protein has three or four regions with amino acid sequence similar to the Bcl-2 protein (Strasser *et al.*, 2000; Crow *et al.*, 2004). These regions are currently known as Bcl-2 homology regions BH1-BH4. Mammalian pro-apoptotic Bcl-2 proteins which promote cell death such as Bax (Bcl-2-associated X protein) and Bak (Bcl-2-antagonist/killer) also contain 2 or 3 BH regions. By contrast, the most potent pro-apoptotic proteins of the Bcl-2 family have only a BH3 region, including Bad (Bcl-2-antagonist of cell death) and tBid (truncated Bid). In addition to the BH regions, many proteins of the Bcl-2 family have a conserved C-terminal transmembrane region which is responsible for their localization to the cytosolic portion of the outer mitochondrial membrane (OMM) which led to the idea, in the "intrinsic" pathway of apoptosis, that anti-apoptotic and pro-apoptotic members of the Bcl-2 family function as transmembrane channels that respectively hinder or promote the efflux of mitochondrial proteins such as cyt c that activate the caspase cascades (Strasser *et al.*, 2000; Jiang and Wang, 2004). Nevertheless, the homo-oligomerization of Bax or Bak on the mitochondrial membrane is regarded as an essential event for the release of cyt c (Wei *et al.*, 2001). It has been reported that either Bax or Bak is required to mediate all events of apoptosis in the intrinsic pathway. The BH3-only proteins such as Bad are also thought to activate Bax or Bak homo-oligomerization (Jiang and Wang, 2004). Interestingly, both pro-apoptotic and anti-apoptotic members of the Bcl-2 family can

physically interact (Oltvai and Korsmeyer, 1994) and their hetero-dimerization is believed to play a key role in regulating the "intrinsic" pathway of apoptosis. The anti-apoptotic members prevent release of mitochondrial proteins by binding to and antagonizing the pro-apoptotic members of the Bcl-2 family (Jiang and Wang, 2004). For instance, Bcl-$_{XL}$ can form a heterodimer with Bak (Sattler *et al.*, 1997).

The activities of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family can be up- or down-regulated by drug-induced gene transcriptions. In the present study, the modulatory effects of CLA-mix and the 9E, 11E-CLA isomer on the apoptosis-regulatory gene expression in the WEHI-3B JCS cells were investigated by the technique of RT-PCR. Briefly, the WEHI-3B JCS cells were either untreated, treated with solvent control (SC), or treated with 150 μM CLA-mix or 150 μM 9E, 11E-CLA for different time periods up to 24 hours. Total RNA were extracted and the apoptosis-regulatory genes of interest were amplified using specific primer pairs. The amount of PCR product was normalized with respect to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

As shown in Fig. 4.8, the expression of the pro-apoptotic gene *Bak* was increased by about 170% while the expression of the anti-apoptotic gene *Bcl-$_{XL}$* was greatly diminished after 24 hours of treatment with CLA-mix. Similarly, Fig. 4.9 shows that the 9E, 11E-CLA isomer increased the expression of the pro-apoptotic gene *Bad* by about 60% and reduced the expression of the anti-apoptotic gene *Bcl-$_{XL}$* by about 80% after 24 and 12 hours of treatment, respectively. In both cases, the expression of the anti-apoptotic *Bcl-$_{XL}$* gene was reduced. However, there was no significant changes in the expression level of *Bad* in the CLA-mix-treated WEHI-3B JCS cells and in the expression level of *Bak* in the 9E, 11E-CLA-treated WEHI-3B JCS cells (data not shown).

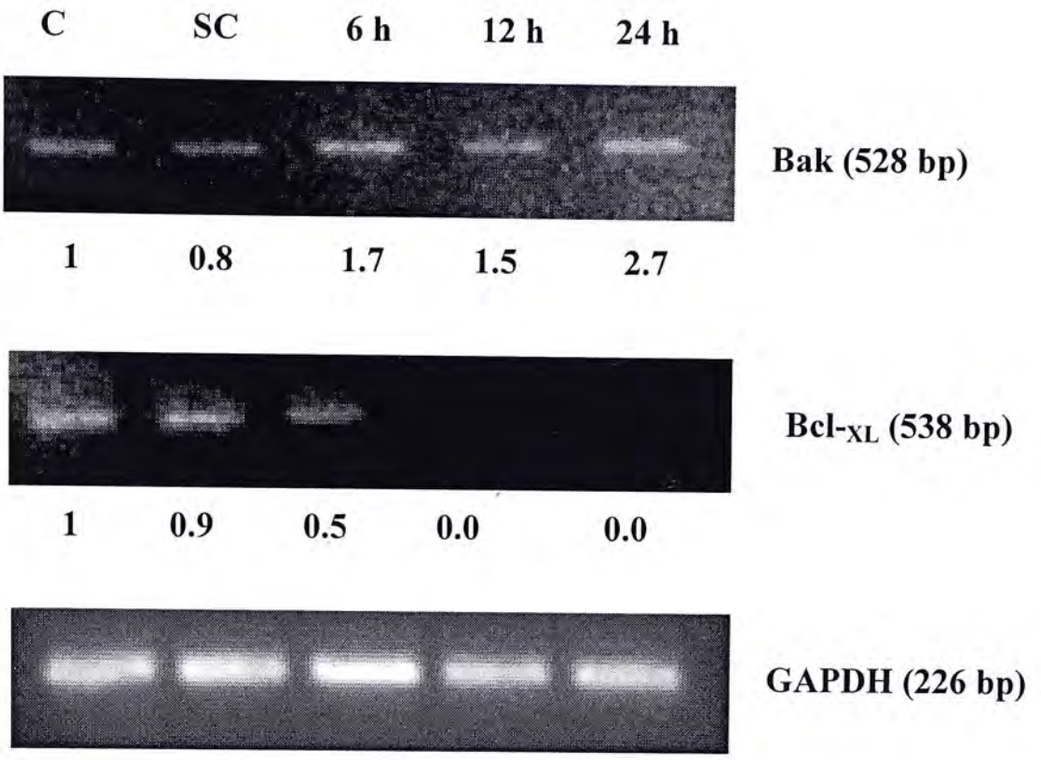



**Fig. 4.8: Analysis of the apoptosis-regulatory gene expression in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by RT-PCR.** WEHI-3B JCS cells ($10^6$ cells) were incubated with 150 μM CLA-mix at 37°C for different time intervals (6, 12 and 24 hours) or treated with medium (C) or ethanol (SC) as controls. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding medium control.

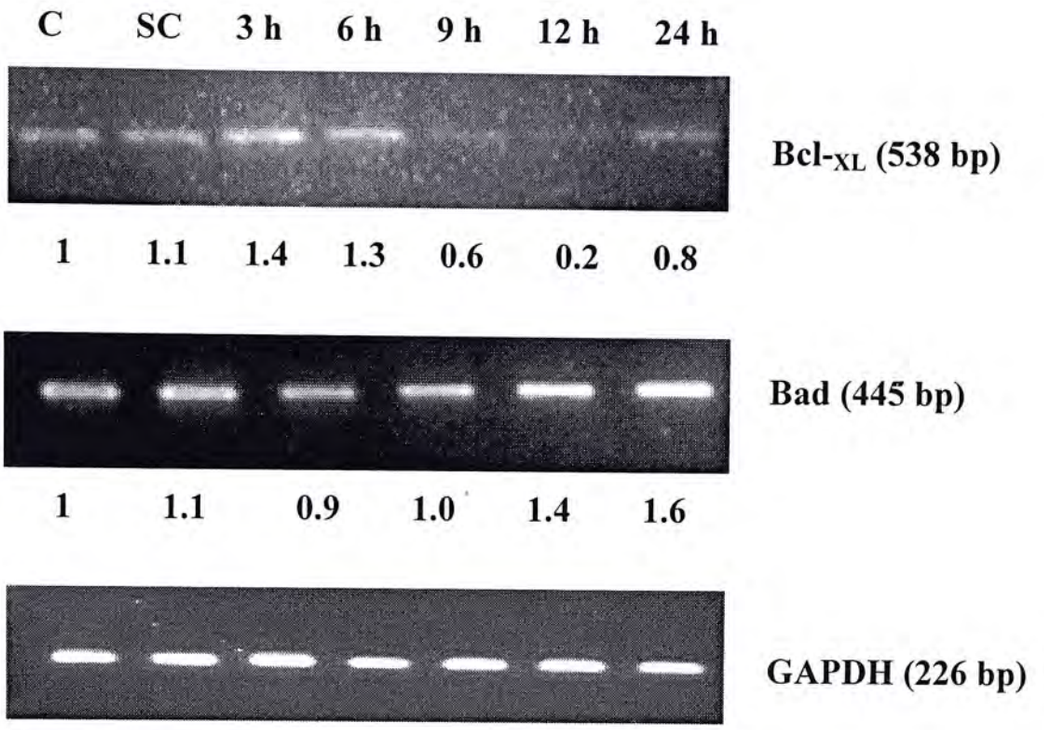



**Fig. 4.9: Analysis of the apoptosis-regulatory gene expression in the 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by RT-PCR.** WEHI-3B JCS cells ($10^6$ cells) were incubated with 150 μM 9E, 11E-CLA at 37°C for different time intervals (3, 6, 9, 12 and 24 hours) or treated with medium (C) or ethanol (SC) as controls. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding medium control.

### 4.2.4 Effect of CLA-mix and its Isomer on the Expression of Apoptosis-regulatory Proteins in the WEHI-3B JCS Cells

It was postulated that the genes which encode molecules for apoptotic signal transduction, including *Fas* and *Fas-L*, could work as tumor-suppressor genes (Nagata, 1999). The identification of Fas (also known as CD95) and Fas ligand (Fas-L) as death receptor and its ligand respectively has facilitated the elucidation of signal transduction through the "extrinsic" pathway. Both Fas and Fas-L belong to the TNF family (Suda *et al.*, 1993). It was found that binding of Fas with Fas-L or cross-linking of Fas with agonistic antibodies induces apoptosis in the Fas-bearing cells.

In addition to the "intrinsic" pathway, it is of interest to know whether CLA can activate the "extrinsic" pathway of apoptosis. Since Fas receptor is a paragon for studying the "extrinsic" pathway, the modulatory effects of CLA-mix and 9E, 11E-CLA on the protein expression of Fas and its ligand, Fas-L, in the WEHI-3B JCS cells were determined by the Western blot analysis. Figures 4.10 and 4.11 show that the Fas and Fas-L proteins were increased by about 2.1- and 2.6-fold respectively after 48 hours of treatment with CLA-mix. Similarly, the Fas and Fas-L proteins were increased by about 2.7- and 3.3-fold respectively after 48 hours of treatment with the 9E, 11E-CLA isomer (Fig. 4.10 and 4.11).

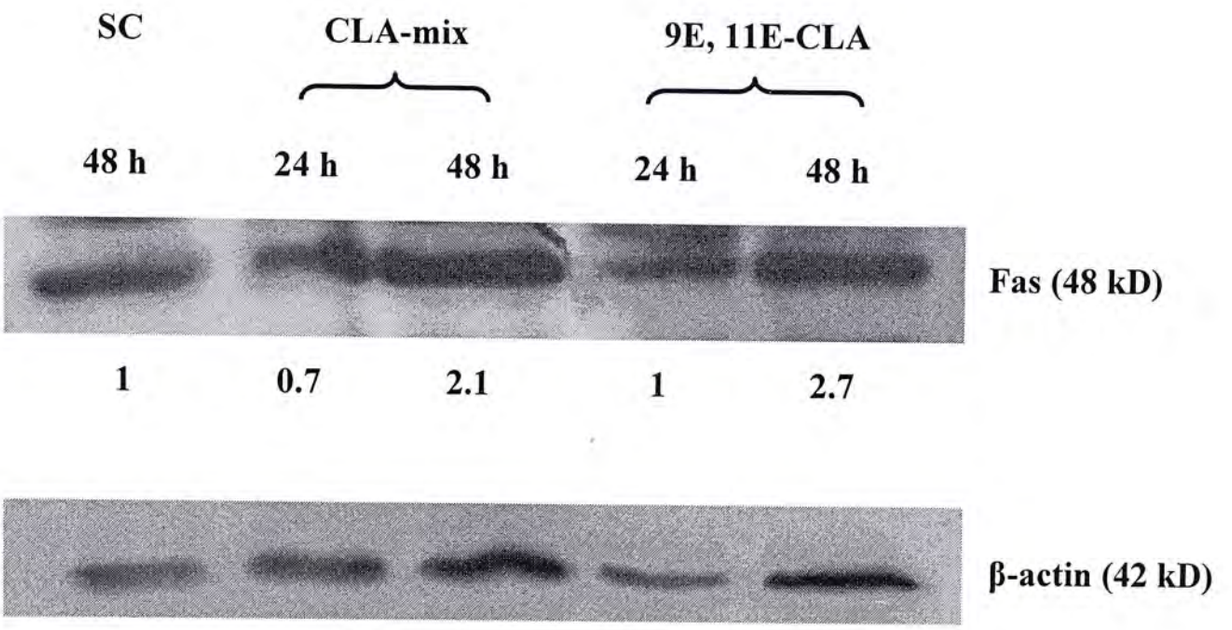



**Fig. 4.10: Analysis of protein expression of Fas in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B cells by Western blot analysis.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with solvent control (0.1% ethanol), 150 μM CLA-mix or 150 μM 9E, 11E-CLA for 24 and 48 hours at 37°C. The proteins of each sample were extracted and analyzed by Western blot as described in Chapter 2. The proteins were probed with specific primary antibody followed by horseradish peroxidase-conjugated secondary antibody and visualized by the ECL assay. The value at the bottom of each band, determined by ImageQuant, represents the relative intensity after normalization with respect to β-actin, and comparison was made with the corresponding solvent control (SC).

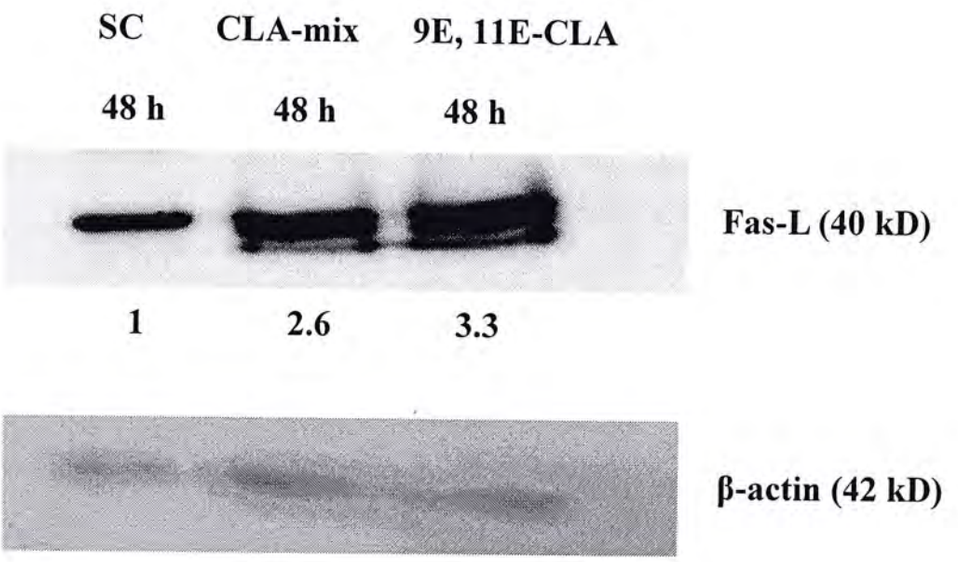



**Fig. 4.11: Analysis of protein expression of Fas ligand (Fas-L) in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B cells by Western blot analysis.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with solvent control (0.1% ethanol), 150 μM CLA-mix or 150 μM 9E, 11E-CLA for 48 hours at 37°C. The proteins of each sample were extracted and analyzed by Western blot as described in Chapter 2. The proteins were probed with specific primary antibody followed by horseradish peroxidase-conjugated secondary antibody and visualized by the ECL assay. The value at the bottom of each band, determined by ImageQuant, represents the relative intensity after normalization with respect to β-actin, and comparison was made with the corresponding solvent control (SC).

### 4.2.5 Effect of CLA-mix and its Isomer on the Induction of Caspase Activity in the WEHI-3B JCS Cells

Accumulated evidence suggests that at least 14 caspases have been identified in mammals (Strasser *et al.*, 2000). The caspases constitute a family of cysteine proteases which cleave target proteins at sites next to aspartic acid residues. Apoptotic caspases are classified as 'initiator' caspases (caspases-2, -8, -9, -10, and -12) or 'effector' caspases (caspases-3, -6, and -7), depending on their point of entry into the apoptotic cascade (Stennicke *et al.*, 1999; Boatright *et al.*, 2003; Crow *et al.*, 2004). Caspases are synthesized as zymogens, also known as procaspases, which have very weak protease activity. The 'initiator' caspases are activated by oligomerization and self-cleavage of their monomeric procaspases, whereas the 'effector' caspases are activated through proteolytic cleavage of their inactive procaspases into active caspases by the upstream activated initiator caspases.

During apoptosis, caspase-9 is a common "initiator" caspase of the "intrinsic" pathway while caspase-8 is a common "initiator" caspase of the "extrinsic" pathway. Once procaspase-9 or -8 is activated into caspase-9 or -8, respectively, "effector" caspases such as caspase-3 will be cleaved and activated accordingly. As shown in Fig. 4.12 to 4.18, the activities of caspases-3, -8 and -9 were significantly enhanced following treatment with CLA-mix or the 9E, 11E-CLA isomer. For caspase-8, the enhanced activity was more prominent after 48 and 24 hours of treatment with CLA-mix and 9E, 11E-CLA respectively in WEHI-3B JCS cells (Fig. 4.12 and 4.13). The caspase-8 activity was increased by 78% and 50% after exposure to CLA-mix and 9E, 11E-CLA respectively for 48 hours (Fig. 4.13). Similarly, for caspase-9, the enhanced activity was more prominent after 24 hours of treatment with CLA-mix or

9E, 11E-CLA. It can be seen that the caspase-9 activity was increased by 33% and 105% after exposure of the WEHI-3B JCS cells to CLA-mix and 9E, 11E-CLA respectively for 24 hours (Fig. 4.15). For caspase-3, the enhanced activity was more prominent after 48 hours of treatment with either CLA-mix or 9E, 11E-CLA in WEHI-3B JCS cells. The caspase-3 activity was enhanced by about 6.4 fold and 8.3 fold after exposure of the WEHI-3B JCS cells to CLA-mix and 9E, 11E-CLA respectively for 48 hours (Fig. 4.17 and 4.18). Moreover, the addition of caspase-specific inhibitors markedly reduced the activity of specific caspase in each case, thus confirming the specificity of each assay in this study.

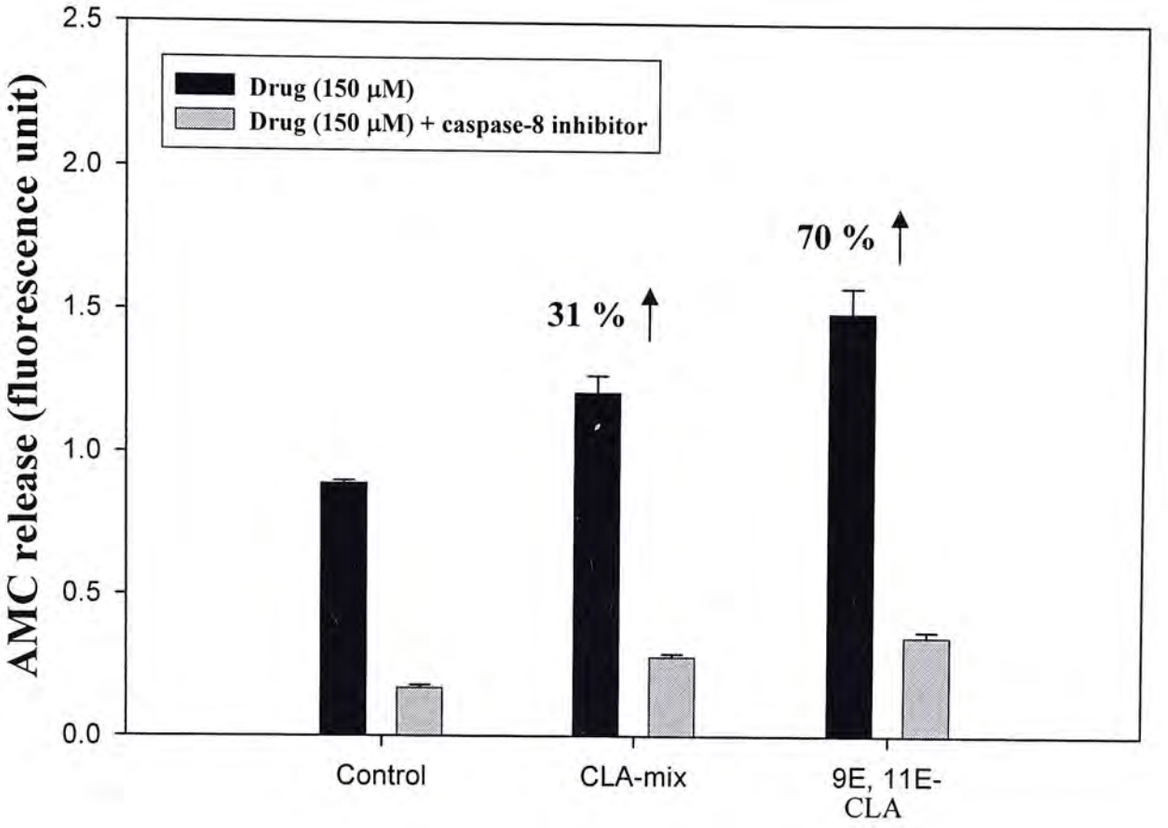



**Fig. 4.12: Analysis of caspase-8 activity in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay after 24 hours of incubation.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM CLA-mix or with 150 μM 9E, 11E-CLA for 24 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-8 (IETD-AMC) with or without its specific inhibitor (IETD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 465 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-8 activity corresponded to the AMC release. The units were quantified by comparing with the AMC standard curve.

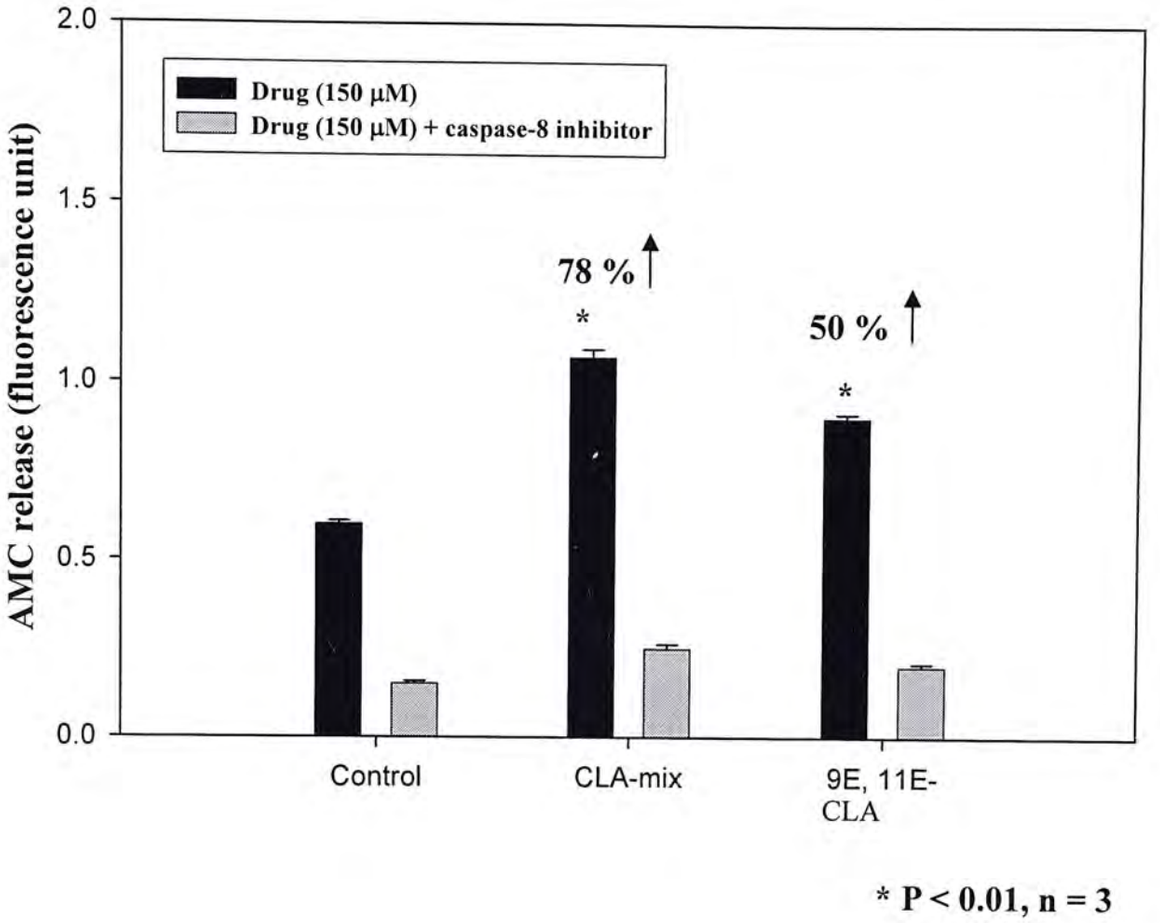



**Fig. 4.13: Analysis of caspase-8 activity in CLA-mix-treated and 9E, 11E-CLA-treated myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay after 48 hours if incubation.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM CLA-mix or with 150 μM 9E, 11E-CLA for 48 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-8 (IETD-AMC) with or without its specific inhibitor (IETD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 465 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-8 activity corresponded to the AMC release. The units were quantified by comparing with the AMC standard curve.

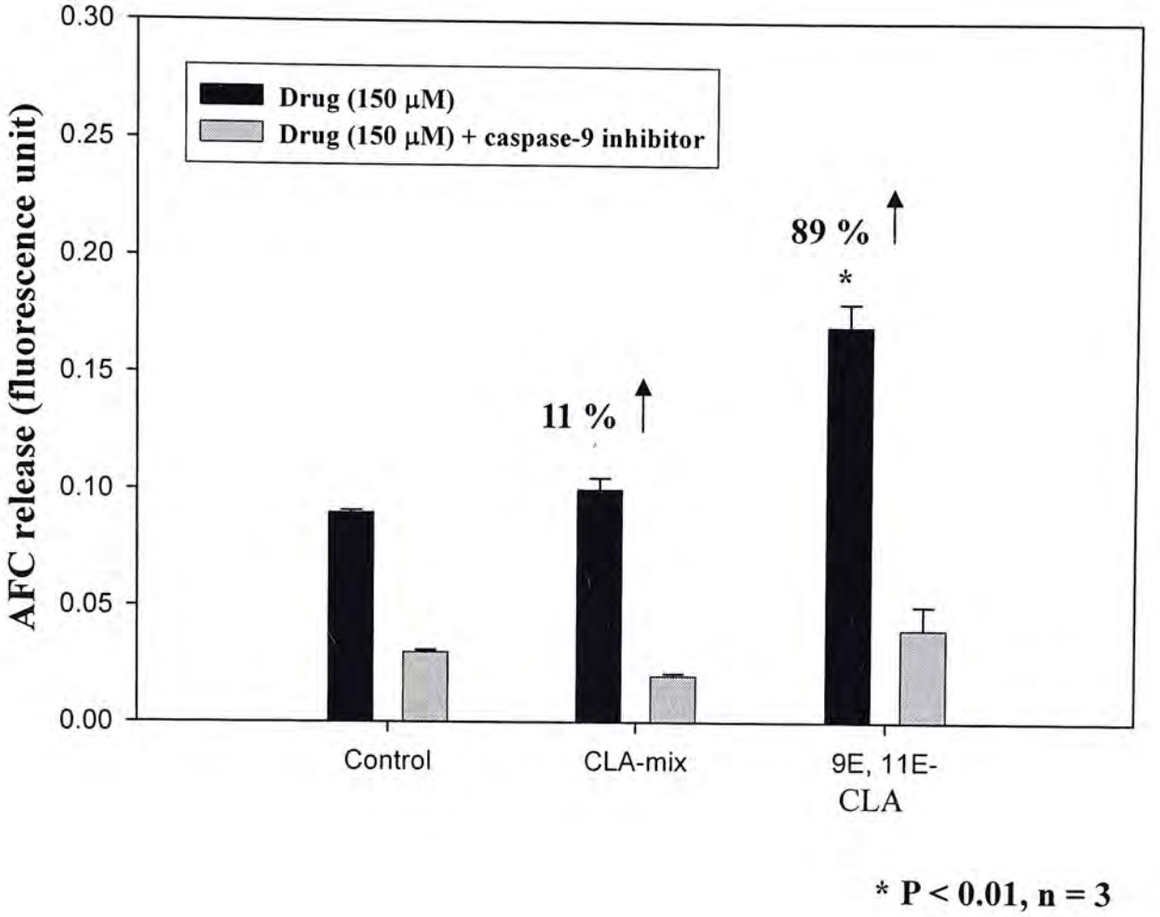



**Fig. 4.14: Analysis of caspase-9 activity in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay after 12 hours of incubation.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM CLA-mix or with 150 μM 9E, 11E-CLA for 12 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-9 (LEHD-AFC) with or without its specific inhibitor (LEHD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 535 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-9 activity corresponded to the AFC release. The fluorescence units were quantified by comparing with the AFC standard curve.

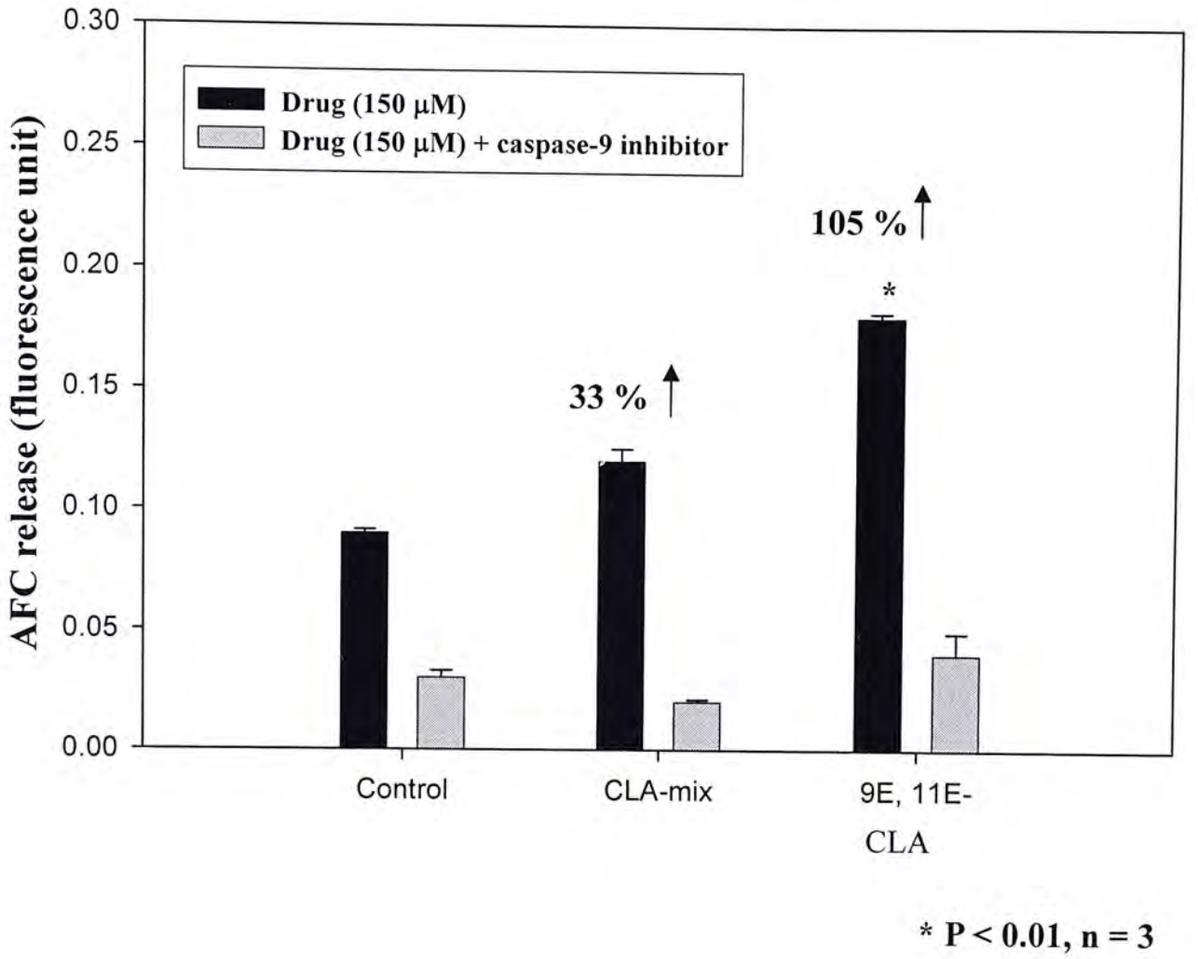



**Fig. 4.15: Analysis of caspase-9 activity in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay after 24 hours of incubation.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM CLA-mix or with 150 μM 9E, 11E-CLA for 24 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-9 (LEHD-AFC) with or without its specific inhibitor (LEHD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 535 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-9 activity corresponded to the AFC release. The fluorescence units were quantified by comparing with the AFC standard curve.

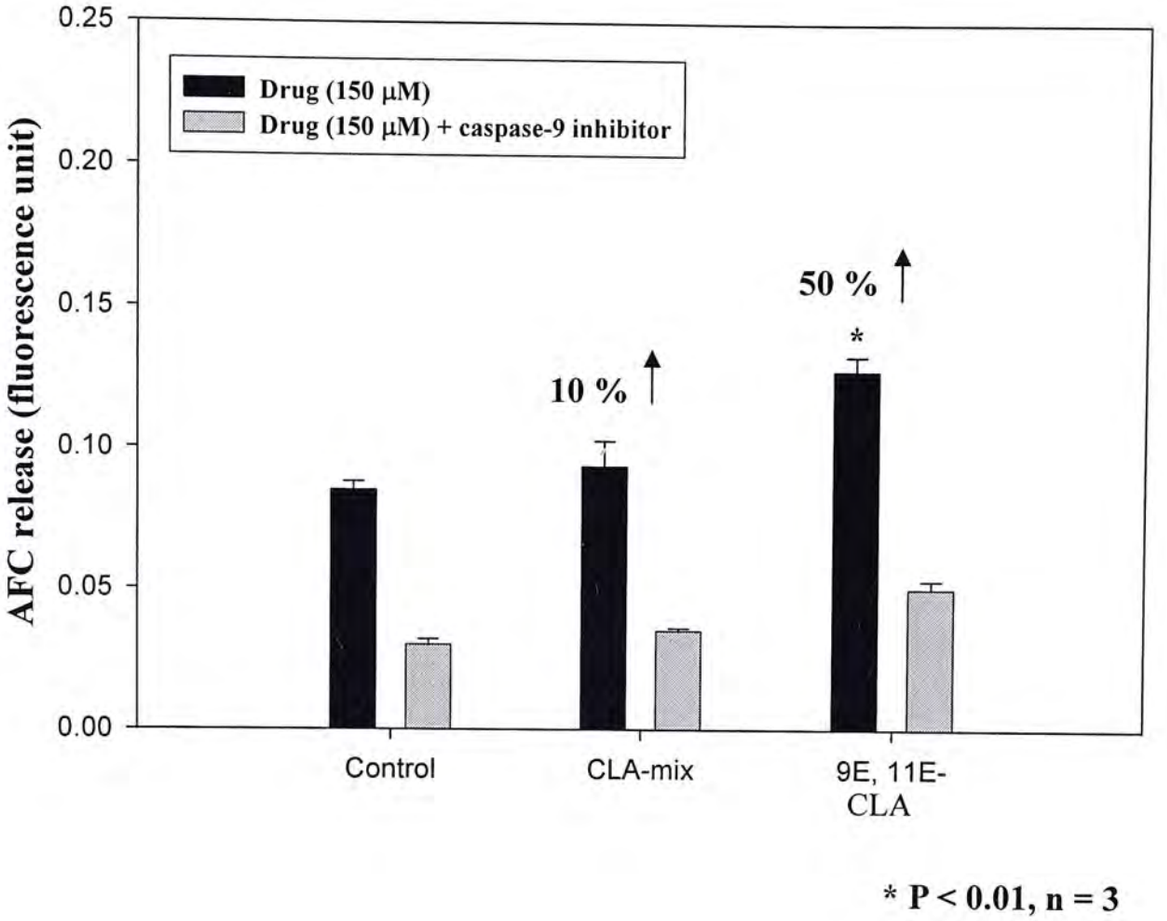



**Fig. 4.16: Analysis of caspase-9 activity in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay after 48 hours of incubation.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM CLA-mix or with 150 μM 9E, 11E-CLA for 48 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-9 (LEHD-AFC) with or without its specific inhibitor (LEHD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 535 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-9 activity corresponded to the AFC release. The fluorescence units were quantified by comparing with the AFC standard curve.

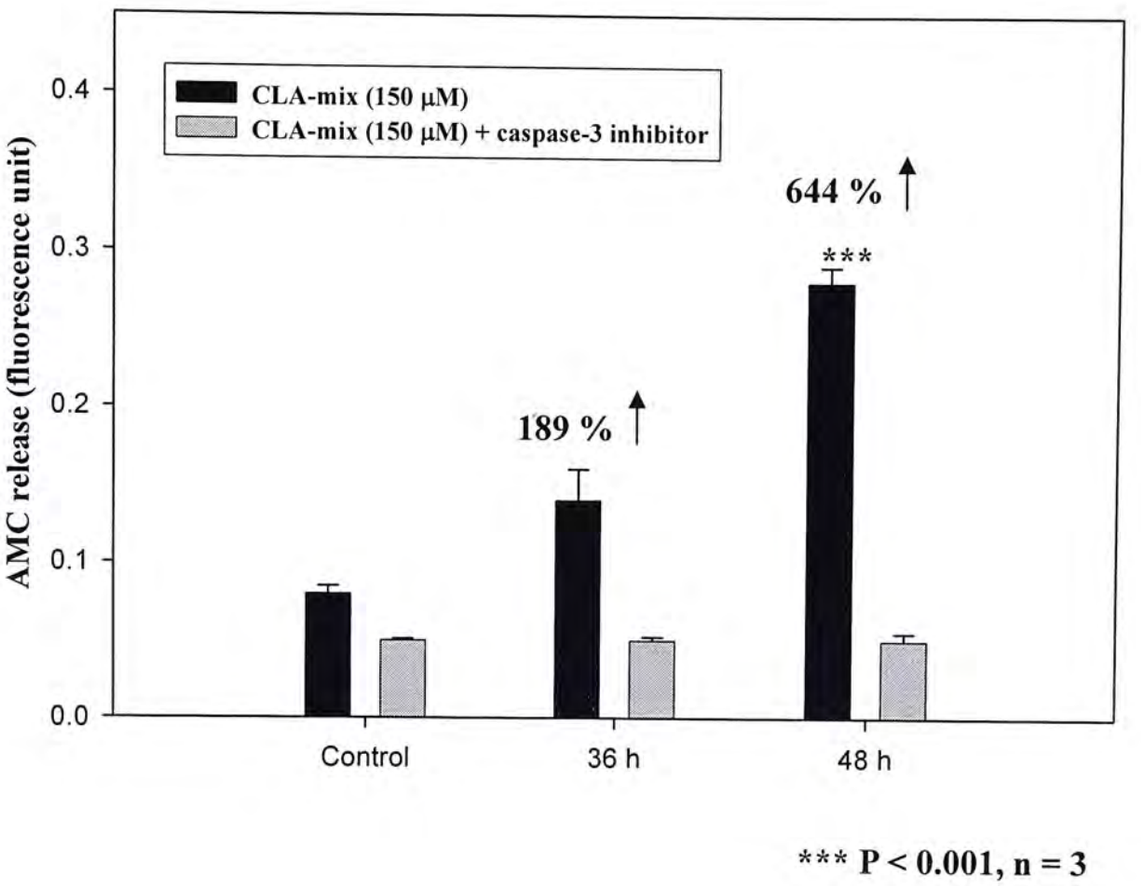



**Fig. 4.17: Analysis of caspase-3 activity in CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 µM CLA-mix for 36 and 48 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-3 (DEVD-AMC) with or without its specific inhibitor (DEVD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 465 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-3 activity corresponded to the AMC release. The fluorescence units were quantified by comparing with the AMC standard curve.

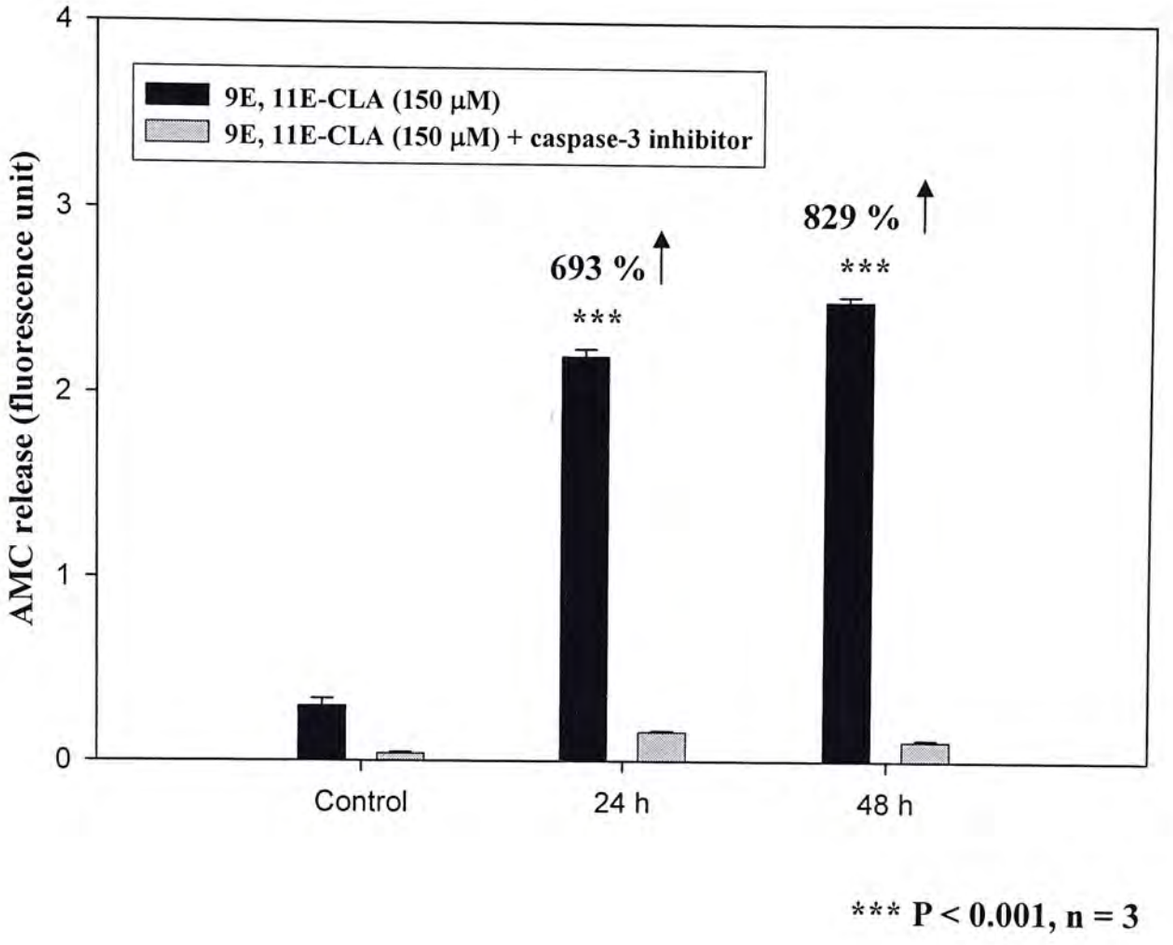



**Fig. 4.18: Analysis of caspase-3 activity in 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells by fluorometric assay.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated either with solvent control or with 150 μM 9E, 11E-CLA for 36 and 48 hours at 37°C. The proteins of each sample were extracted and incubated with specific substrate of caspase-3 (DEVD-AMC) with or without its specific inhibitor (DEVD-CHO). The samples were subjected to excitation at 430 nm and the fluorescence emitted at 465 nm was measured by the fluorescence plate reader, Cytofluo. The caspase-3 activity corresponded to the AMC release. The fluorescence units were quantified by comparing with the AMC standard curve.

### 4.2.6 Effect of CLA-mix and its Isomer on the Induction of ROS in the WEHI-3B JCS Cells

Reactive oxygen species (ROS) have been previously implicated in the induction or enhancement of apoptosis (Huang *et al.*, 2003), which are produced upon stress stimulation such as exposure to UV, γ-irradiation, or cytotoxic drugs. Mitochondria are a major source and target of ROS (Droge, 2002). Excessive ROS crucial for apoptosis are raised as a consequence of electron leakage from the respiratory chain complexes I and III in the inner mitochondrial membranes (IMM). For instance, superoxide anion ($O_2^-$) is formed when an electron is captured by molecular oxygen (Hileman *et al*, 2004). Yet, the mechanisms responsible for ROS-mediated apoptosis remain poorly understood. It is now thought that ROS can trigger cyt c release in mitochondrial permeability transition (MPT)-dependent or -independent manner. ROS may promote MPT through oxidation of thiol groups on the adenine nucleotide translocator (ANT) of IMM (Kanno *et al.*, 2004). However, the mechanism by which cyt c is released through the outer mitochondrial membrane (OMM) in this case is not clear.

Previous investigation showed that dietary CLA supplement had prooxidant activity (Basu *et al.*, 2000). Therefore, the production of ROS in CLA-treated WEHI-3B JCS cells was analyzed. Superoxide anion is a major ROS component in the mitochondria (Ito *et al.*, 2004). Intracellular $O_2^-$ generation is measured by the oxidation of dihydroethidium to fluorescent ethidium. In this study, it was found that CLA-mix and the 9E, 11E-CLA isomer enhanced the $O_2^-$ production in the WEHI-3B JCS cells (Fig. 4.19 and 4.20). More drastic production of $O_2^-$ could be detected, in both cases, after 12 hours of CLA treatment.

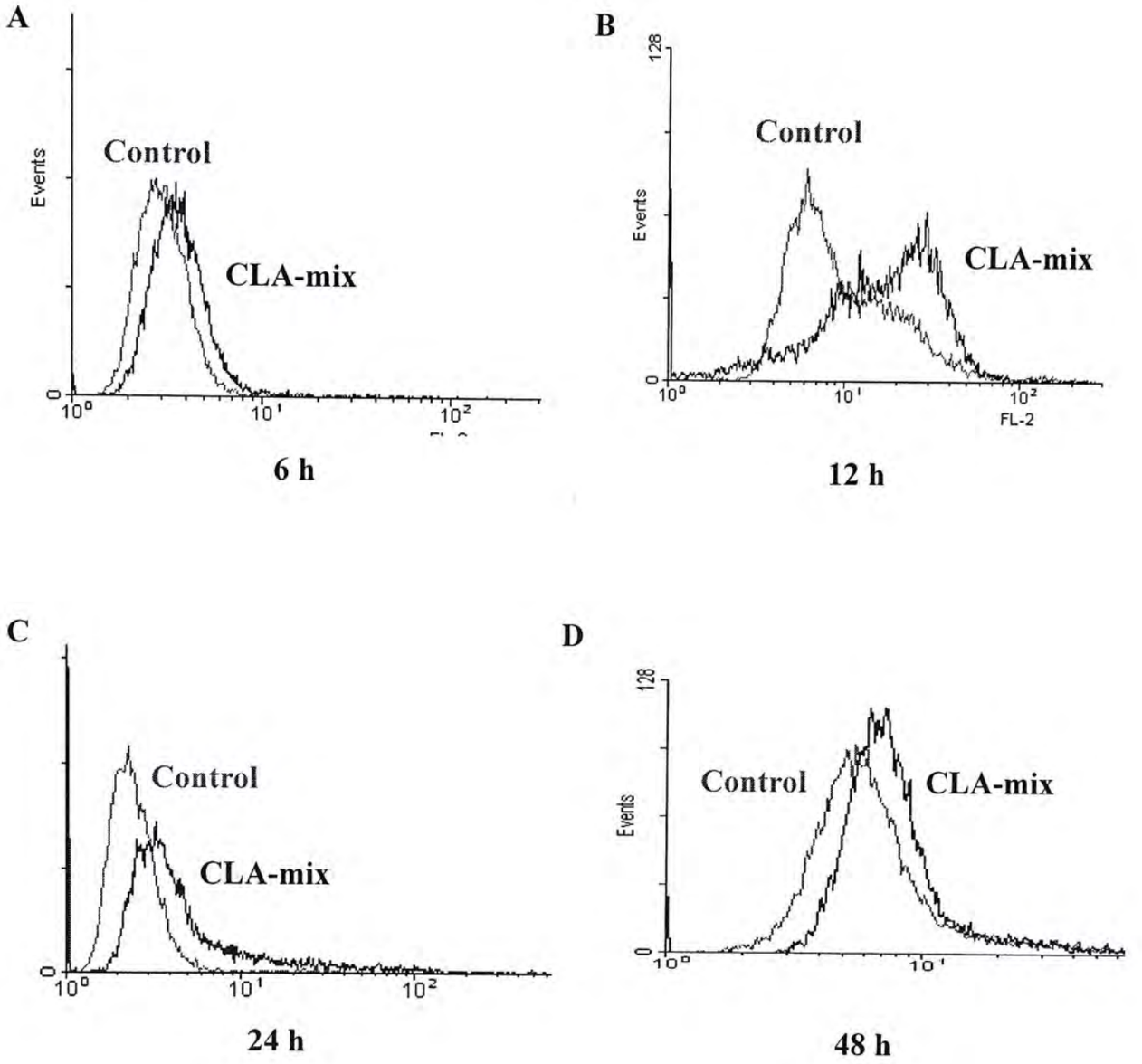



**Fig. 4.19: Analysis of superoxide anion accumulation in CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with 150 µM CLA-mix for (A) 6 hours, (B) 12 hours, (C) 24 hours, or (D) 48 hours. The induced cells were then cultured with dihydroethidium and the fluorescence was measured by flow cytometry. The fluorescence in CLA-mix-treated cells was compared to the solvent-treated cells (control) in each case.

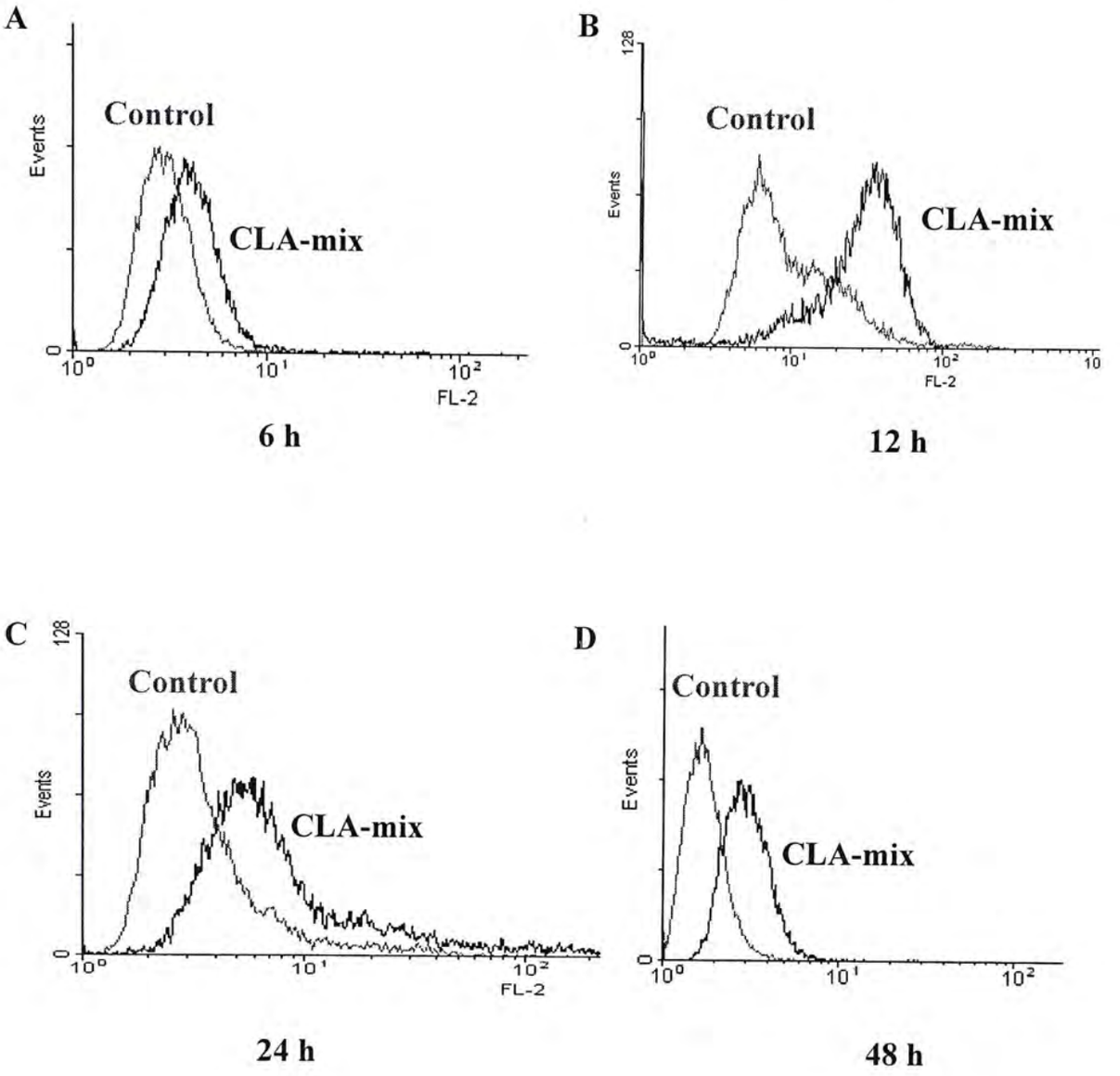



**Fig. 4.20: Analysis of superoxide anion accumulation in 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were treated with 150 μM 9E, 11E-CLA for (A) 6 hours, (B) 12 hours, (C) 24 hours, or (D) 48 hours. The induced cells were then cultured with dihydroethidium and the fluorescence was measured by flow cytometry. The fluorescence in CLA-mix-treated cells was compared to the solvent-treated cells (control) in each case.

### 4.2.7 Effect of Antioxidants on the Induction of ROS by CLA-mix and its Isomer in the WEHI-3B JCS Cells

Since intracellular accumulation of ROS causes detrimental damages, such as lipid peroxidation, DNA adduct formation, protein oxidation, and enzyme inactivation, ultimately leading to cell death (Hileman *et al.*, 2004), therefore, the body cells have inherited a highly regulated antioxidant defense system to maintain proximal ROS levels. Primary ROS, such as $O_2^-$, diffuse into the cytoplasm and are scavenged by the antioxidants. This prevents partly the formation of secondary radicals like hydroxyl radical ($^{\cdot}OH$) by $Fe^{2+}$-catalyzed Fenton reaction (Tsuruga *et al.*, 2003).

Superoxide anion is converted rapidly to $H_2O_2$ by superoxide dismutase (SOD). Most of the $H_2O_2$ generated was further converted to $H_2O$ by catalase and glutathione peroxidase. In this study, we investigated the effects of specific antioxidants, such as SOD and N-acetylcysteine (NAC), on CLA-induced ROS generation. As shown in Fig. 4.21 and 4.22, preincubation with 200 units/ml SOD for 3 hours or 15 mM NAC for 2 hours significantly reduced the CLA-induced $O_2^-$ production in the CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells after 24 hours of treatment.

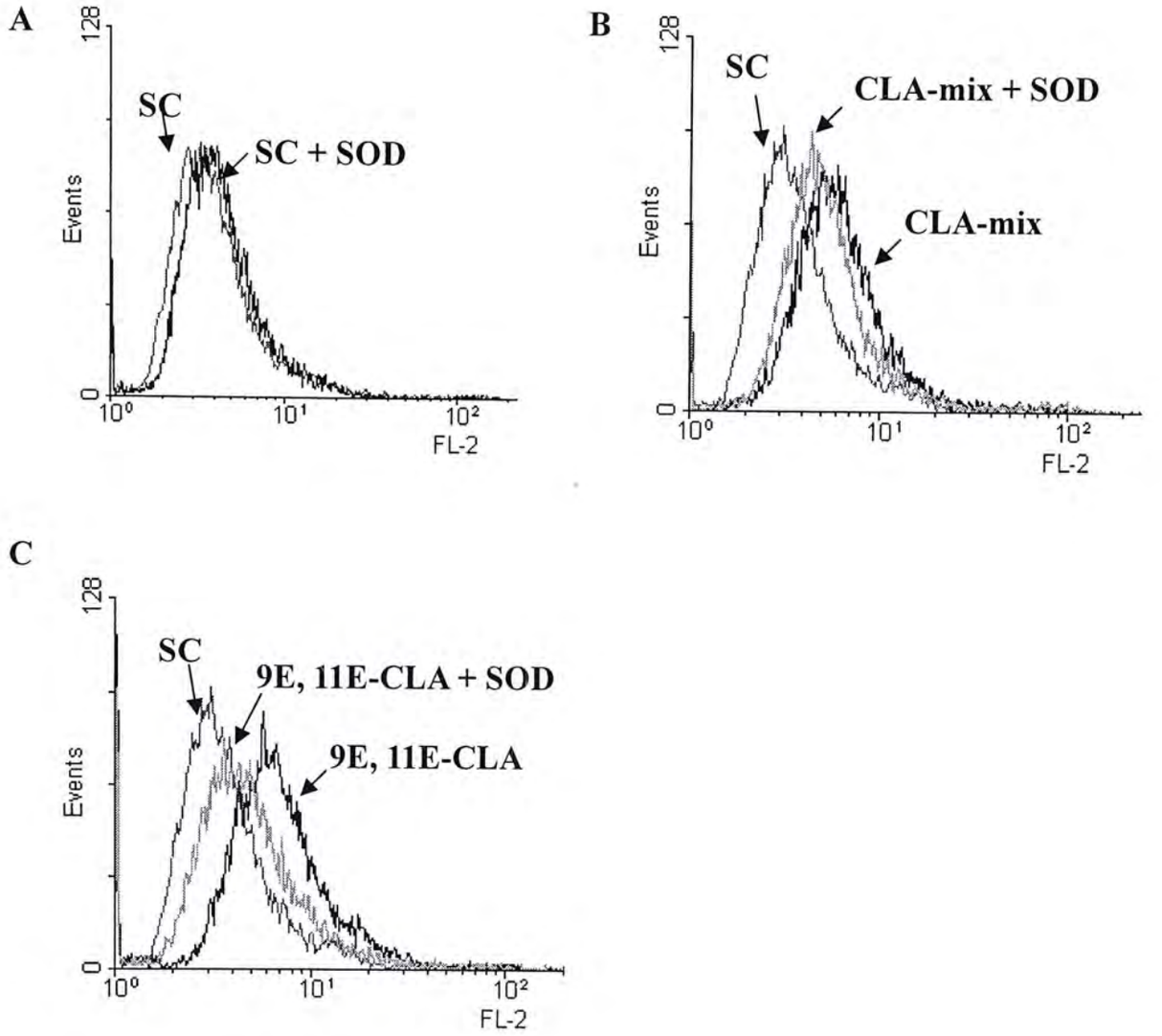



**Fig. 4.21: Effects of superoxide dismutase on superoxide anion production in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were preincubated with 200 units/ml SOD for 3 hours (A-C), and then cultured with (A) solvent control (SC), (B) 150 μM CLA-mix, or (C) 150 μM 9E, 11E-CLA for another 24 hours. The induced cells were then treated with dihydroethidium and analyzed by flow cytometry.

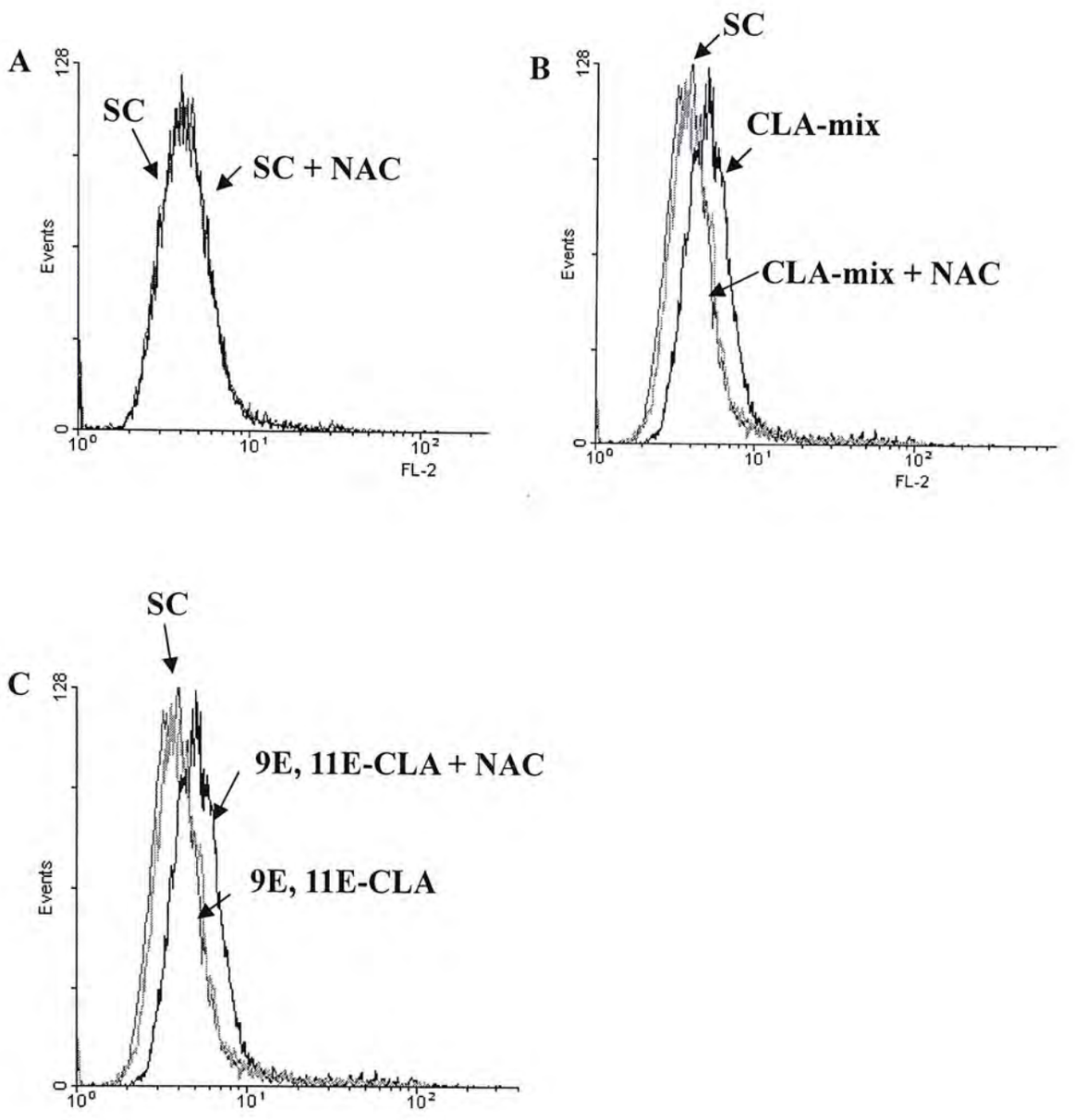



**Fig. 4.22: Effects of N-acetylcysteine (NAC) on superoxide anion production in CLA-mix treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were preincubated with 15 mM NAC for 2 hours (A-C), and then cultured with (A) solvent control (SC), (B) 150 μM CLA-mix, or (C) 150 μM 9E, 11E-CLA for another 24 hours. The induced cells were then treated with dihydroethidium and analyzed by flow cytometry.

### 4.2.8 Effect of Antioxidants on the Induction of Apoptosis by CLA-mix and its Isomer in the WEHI-3B JCS Cells

As demonstrated in section 4.2.7, antioxidants such as SOD and NAC could significantly reduce the CLA-induced $O_2^-$ production in the CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells. Therefore, it was of interest to know whether these antioxidants could also reduce the CLA-induced DNA fragmentation in WEHI-3B JCS cells. The results showed that DNA fragmentation triggered by CLA-mix and 9E, 11E-CLA could be partly diminished by prior treatment with either SOD (Fig. 4.23) or NAC (Fig. 4.24). However, the antioxidants could not completely block the CLA-induced DNA fragmentation, and this suggests that other apoptosis signaling pathways, such as those regulated by the Bcl-2 family members or the death receptor Fas, may be involved in triggering the apoptosis of the CLA-treated WEHI-3B JCS cells.

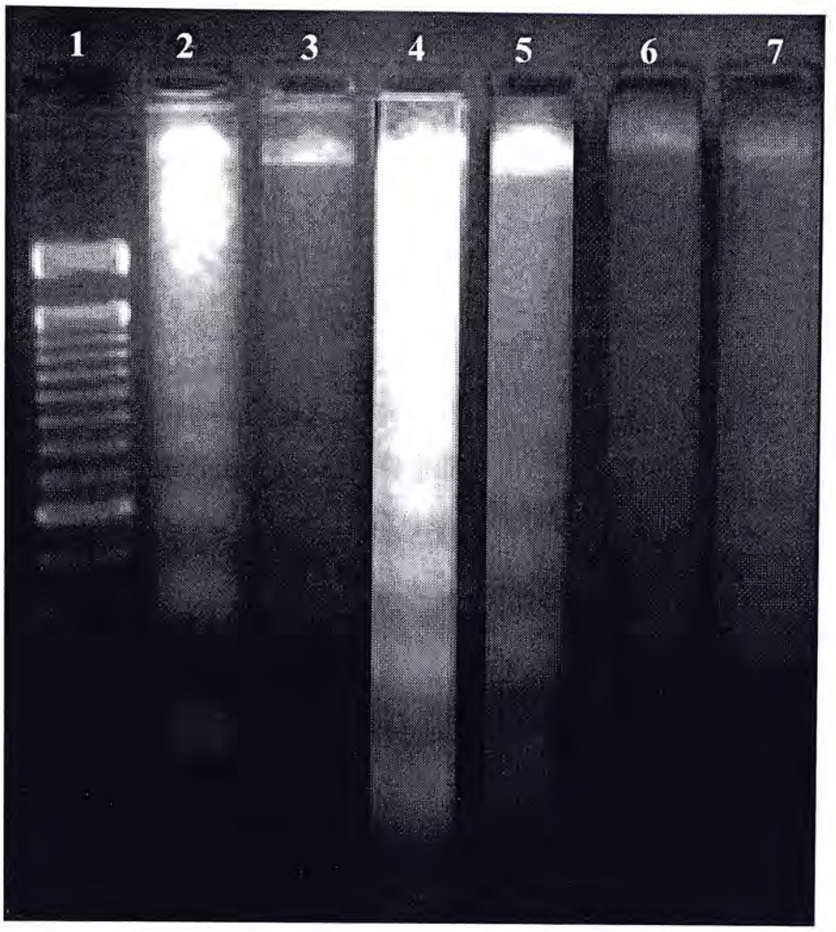



**Fig. 4.23: Effects of superoxide dismutase on induction of DNA fragmentation in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated (Lanes 2, 4 and 6) or pretreated with SOD (200 units/ml) for 3 hours (Lanes 3, 5 and 7). The cells were then cultured with solvent control (0.1% ethanol) (Lanes 6 and 7), 150 μM CLA-mix (Lanes 2 and 3) or 9E, 11E-CLA (Lanes 4 and 5) at 37°C for 48 hours. Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA marker
Lane 2: JCS cells cultured with 150 μM CLA-mix
Lane 3: JCS cells cultured with 150 μM CLA-mix (with SOD pretreatment)
Lane 4: JCS cells cultured with 150 μM 9E, 11E-CLA
Lane 5: JCS cells cultured with 150 μM 9E, 11E-CLA (with SOD pretreatment)
Lane 6: JCS cells cultured with solvent control
Lane 7: JCS cells cultured with solvent control (with SOD pretreatment)

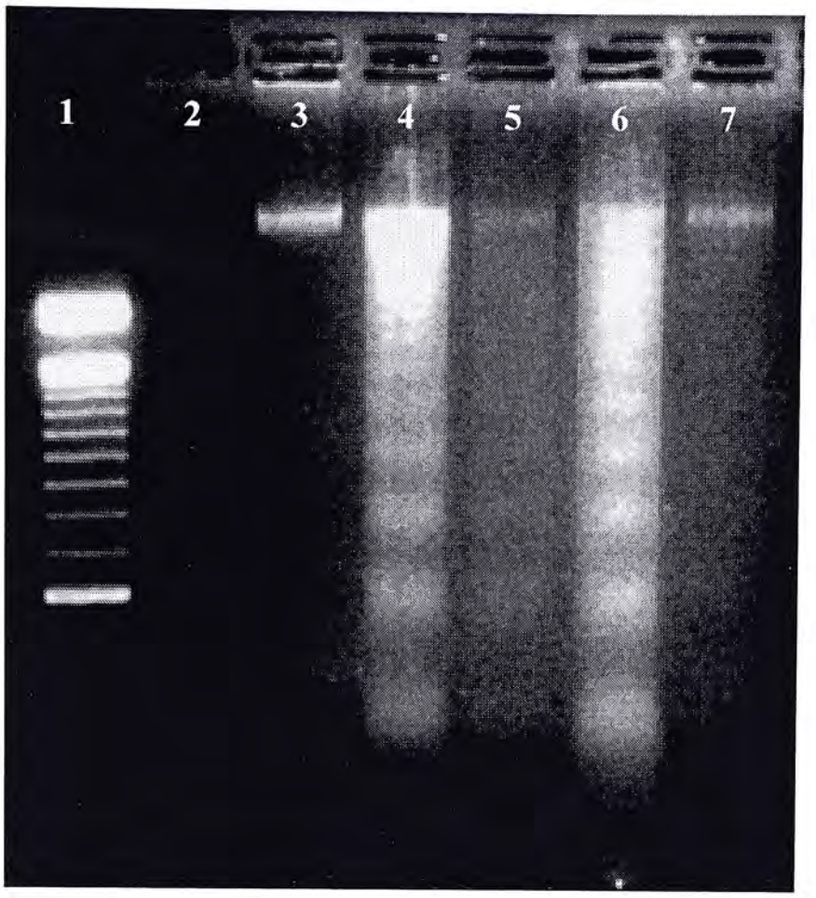



**Fig. 4.24: Effects of N-acetylcysteine on induction of DNA fragmentation in CLA-mix-treated and 9E, 11E-CLA-treated murine myelomonocytic leukemia WEHI-3B cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were either untreated (Lanes 2, 4 and 6) or pretreated with NAC (15 mM) for 2 hours (Lanes 3, 5 and 7). The cells were then cultured with solvent control (0.1% ethanol) (Lanes 2 and 3), 150 μM CLA-mix (Lanes 4 and 5) or 9E, 11E-CLA (Lanes 6 and 7) at 37°C for 48 hours. Apoptotic DNA fragments were extracted by mild detergent IGEPAL CA-630 lysis buffer, and were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Lane 1: 100 bp DNA marker
Lane 2: JCS cells cultured with solvent control
Lane 3: JCS cells cultured with solvent control (with NAC pretreatment)
Lane 4: JCS cells cultured with 150 μM CLA-mix
Lane 5: JCS cells cultured with 150 μM CLA-mix (with NAC pretreatment)
Lane 6: JCS cells cultured with 150 μM 9E, 11E-CLA
Lane 7: JCS cells cultured with 150 μM 9E, 11E-CLA (with NAC pretreatment)

## 4.3 Discussion

One of the most established approaches to cancer chemotherapy is the induction of DNA damage and subsequent induction of apoptosis (Johnson and Walker, 1999). To date, several studies have revealed the ability of CLA, especially the *trans*-10, *cis*-12- (10E, 12Z-) CLA isomer, to induce apoptosis in a variety of cancer cell lines (Palombo *et al.*, 2002; Yamasaki *et al.*, 2002; Oh *et al.*, 2003; Maggiora *et al.*, 2004; Ochoa *et al.*, 2004; Kim *et al.*, 2005). Therefore, in this chapter, investigation on whether CLA could trigger apoptosis of the murine myelomonocytic leukemia WEHI-3B JCS cells was carried out. Our results showed that CLA-mix and two of the CLA isomers, *trans*-10, *cis*-12 (10E, 12Z-) CLA and *trans*-9, *trans*-11 (9E, 11E-) CLA, were able to trigger apoptosis in WEHI-3B JCS cells while the other two CLA isomers, *cis*-9, *trans*-11 (9Z, 11E-) CLA and *cis*-9, *cis*-11 (9Z, 11Z-) CLA, failed to do so. In fact, this is the first report documenting that the 9E, 11E-CLA isomer is a more potent inducer of apoptosis on tumor cells than other CLA isomers so far investigated. Moreover, we had shown that the CLA apoptosis of WEHI-3B JCS cells was not detected until 48 hours after treatment, as measured by the DNA fragmentation assay and the cell death detection ELISA kit.

Our findings also demonstrated that both CLA-mix and the 9E, 11E-CLA isomer could induce mitochondrial membrane depolarization in the WEHI-3B JCS cells. Previous reports have shown that disturbances leading to loss of the mitochondrial membrane potential ($\Delta\Psi m$) include formation of mitochondrial permeability transition (MPT) by pro-apoptotic Bcl-2 family proteins, such as Bax, Bak, and Bad, during the "intrinsic" pathway of apoptosis (Terrones *et al.*, 2004). Although it is believed that the anti-apoptotic members prevent efflux of mitochondrial proteins by binding to and antagonizing the pro-apoptotic members of the Bcl-2 family, both

Bcl-2 and Bcl-$_{XL}$ may exert their anti-apoptotic effects through mechanisms other than direct interactions with Bax and Bak (Crow *et al.*, 2004). They can function as "sinks" that sequester the BH3-only pro-apoptotic proteins, such as Bad, preventing them from activating Bax and Bak (Cheng *et al.*, 2001). Moreover, Bcl-$_{XL}$ has been shown to interact with the adaptor protein apoptotic protease-activating factor (Apaf-1) to prevent it from activating procaspase-9 in the "intrinsic" pathway (Pan *et al.*, 1998). Since the pro-apoptotic members of the Bcl-2 family can bind to the anti-apoptotic members, such hetero-dimerization frees Apaf-1 to activate procaspase-9. However, in the "extrinsic" pathway, the Fas-associated protein with death domain (FADD)-induced activation of caspase-8 is not blocked by anti-apoptotic Bcl-2 family members (Martin *et al.*, 1998).

Therefore, in order to elucidate the possible mechanisms by which CLA-mix and the 9E, 11E-CLA exerted their pro-apoptotic activities on the WEHI-3B JCS cells, their effects on the expression of the Bcl-2 family genes were first studied. From the gene expression study of CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells, it was found that the anti-apoptotic *Bcl-x$_L$* gene was down-regulated while the pro-apoptotic *Bak* and *Bad* genes were up-regulated. Nevertheless, in addition to transcriptional activation, the activity of different pro-apoptotic Bcl-2 family members is controlled by post-translational modifications. For instances, the activity of Bad can be regulated by protein kinase A-mediated phosphorylation (Harada *et al.*, 1999). Moreover, both Bcl-2 and Bcl-x$_L$ have been also shown to be regulated by phosphorylation (Ito *et al.*, 1997; Chang *et al.*, 1997).

Apart from modulating the expression of the apoptosis-regulatory genes by CLA and its isomer, our results showed that the protein expression of both Fas and Fas-L was up-regulated in CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells. These

findings suggest that apoptosis triggered by CLA-mix and the 9E, 11E-CLA isomer is signaled through both the "intrinsic" and "extrinsic" pathways. This was further confirmed by studies on the activities of caspases-3, -8 and -9. It was found that both CLA-mix and the 9E, 11E-CLA isomer respectively increased the activities of "initiator" caspases-8 and -9 as well as "effector" caspase-3 in WEHI-3B JCS cells. Our results are in line with a previous report showing that the CLA, particularly the 10E, 12Z-CLA isomer, can induce caspase-dependent apoptosis in human colorectal (MIP-101) and prostate (PC-3) carcinoma cells (Palombo *et al.*, 2002). Moreover, Yamasaki *et al.* (2002) also demonstrated that the 10E, 12Z-CLA but not the 9Z, 11E-CLA isomer induced apoptosis of rat hepatoma dRLh-84 cells through activation of caspases-3 and -9.

Caspases-9 and -8 are initiators of the "intrinsic" and "extrinsic" pathways of apoptosis, respectively. In the "intrinsic" pathway, mitochondria seem to be the main cellular reservoir of procaspase-9. In the presence of ATP or dATP, cyt c binds to an adaptor protein, Apaf-1, which recruits procaspase-9 to form an apoptosome (Earnshaw, 1999). Apaf-1 recruits procaspase-9 via its N-terminal caspase-activation recruitment domain (CARD). In its quiescent state, Apaf-1 is a compact molecule. In the presence of ATP or dATP, cyt c displaces the CARD domain, allowing the compact structure of Apaf-1 to stretch out into a more linear molecule to form apoptosome with procaspase-9 (Jiang and Wang, 2000). This leads to autocatalytic activation of procaspase-9 to caspase-9. Activated caspase-9 cleaves and activates the downstream procaspase-3, which is also recruited into the apoptosome (Bratton *et al.*, 2001).

On the other hand, in the "extrinsic" pathway, caspase-8 is activated when the death receptor Fas is activated by oligomerization (Nagata, 1999). Fas-L induces

trimerization of Fas and the trimerized cytoplasmic region of Fas then transduces the death signal. When Fas is activated, an adaptor molecule called FADD is recruited to Fas and binds to Fas via interactions between the death domains at its C terminus (Boldin *et al.*, 1996). The N terminus of FADD, known as the death-effector domain (DED), is responsible for recruiting procaspase-8 (Muzio *et al.*, 1996). As a result, the DISC (death-inducing signaling complex) is formed which leads to oligomerization, self-cleavage, and activation of procaspase-8 (Nagata, 1999). Activated caspase-8 then cleaves and activates downstream effector caspases such as caspases-3 and -7. In fact, caspase-8 also functions as a direct conduit between the "extrinsic" and "intrinsic" pathways by cleaving and activating Bid, a pro-apoptotic member of the Bcl-2 family. Activated caspase-8 cleaves Bid into truncated Bid (tBid) to expose its BH3 domain to the cytosol (Cheng *et al.*, 2003; Crow *et al.*, 2004). After cleavage, tBid translocates to the mitochondria and inserts into the OMM. Bid-induced permeabilization of the OMM requires the BH3 domain of tBid as well as Bak or Bax. The exposed BH3 domain of tBid can bind to Bak, leading to the displacement of VDAC2 from Bak. Similarly, the BH3 domain of Bid also binds to Bax, facilitating its insertion into the OMM to induce cyt c release and the subsequent cyt c-mediated activation of caspases such as caspases-9 and -3 in the "intrinsic" pathway.

During apoptotic execution, effector caspases cleave and inactivate certain vital cellular proteins, including DNA repair enzymes, gelsolin, lamin, p53 inhibitor MDM2, and protein kinase Cδ (Strasser *et al.*, 2000). Therefore, effector caspases act both as executioners that cleave key proteins to promote death activities, and as executives that turn the cell-survival pathways off (Earnshaw, 1999). One such death-promoting factor is a nuclease called caspase-activated DNase (CAD) (Enari *et al.*, 1998), also known as caspase-activated nuclease. CAD is ubiquitously expressed

in various tissues and is normally inactivated by binding to its inhibitor iCAD, also called DNA fragmentation factor-45 (DFF-45) (Enari *et al.*, 1998; Sakahira *et al.*, 1998; Tang and Kidd, 1998). During apoptosis, iCAD is cleaved by effector caspases-3 and -7, leading to the release of active CAD which then cleaves chromosomal DNA into fragments of 50-200 kbp. Moreover, CAD can induce the collapse and hypercondensation of the chromatin against the nuclear periphery (Enari *et al.*, 1998).

Growing evidence has shown that intrinsic oxidative stress in malignant cells offers therapeutic selectivity for cancer chemotherapy. Cancer cells are more active in the production of $O_2^-$ than normal cells and are thereby more vulnerable to be destroyed by reactive oxygen species (ROS)-generating agents (Hileman *et al.*, 2004). ROS is a collective term for free radicals such as superoxide anions ($O_2^-$), hydroxyl radicals ($OH^-$), and some other non-radical oxygen derivatives such as hydrogen peroxide ($H_2O_2$) (Gorman *et al.*, 1997). Of which, $O_2^-$ have been well-characterized as apoptotic inducers in a variety of cell types including leukemia cells (Fuchs *et al.*, 1994; Zhou *et al.*, 2003; Cam *et al.*, 2004). In our study, we found that CLA-mix and the 9E, 11E-CLA isomer enhanced $O_2^-$ production in the WEHI-3B JCS cells after 12 hours of incubation. Therefore, the CLA-induced oxidative stress in our study is consistent with other groups showing the prooxidant activity of dietary CLA supplement (Basu *et al.*, 2000).

Mitochondria are the site where many ROS-metabolizing enzymes are situated, including superoxide dismutase (SOD), catalase, and various peroxidases. Therefore, high level of ROS accumulation may be due to the loss of feed-back inhibition of the respiratory chain after depletion of cellular ATP (Swamy and Huat, 2003). In the present study, we demonstrated that antioxidants such as SOD and NAC could reduce

the CLA-induced ROS production as well as the formation of DNA fragmentation in WEHI-3B JCS cells. SOD is the key enzyme required to remove $O_2^-$ by converting it to $H_2O_2$, which is further eliminated by catalase and peroxidases (Fridovich *et al.*, 1995). NAC, a precursor of reduced glutathione (GSH), is also capable of quenching ROS. Prior treatment with either 200 units/ml SOD or 15 mM NAC significantly reduced the production of $O_2^-$ and partly blocked apoptosis in both CLA-mix-treated and 9E, 11E-CLA-treated WEHI-3B JCS cells. As a consequence, it could be speculated that accumulation of CLA-induced ROS in WEHI-3B JCS cells may be due to inhibition of intracellular antioxidant defense system. Intracellular GSH is a known substrate or cofactor of protective enzymes. GSH controls the redox state of a cell by quenching ROS and keeping the enzyme GSH peroxidase in a reduced state (Sies, 1999). Accumulating evidence showed that early GSH depletion caused mitochondrial membrane depolarization with simultaneously ROS generation prior to induction of apoptosis (Swamy and Huat, 2003). A recent study also demonstrated that upon exposure to CLA, GSH depletion and reduced GSH peroxidase activity were found in the leukemia Jurkat T cells (Bergamo *et al.*, 2004) and in pancreatic intratumoral tissue (Kilian *et al.*, 2003), respectively.

In response to DNA damage, the p53 protein triggers the onset of DNA repair leading to the completion of cell cycle, or induces apoptosis leading to the exit from the cell cycle (Offer *et al.*, 2002). High level of p53 was found to induce apoptosis in human promyelocytic leukemia HL-60 cells (Ronen *et al.*, 1996). Marchenko *et al* (2000) found that p53 protein localizes to mitochondria at the onset of p53-dependent apoptosis. The tumor suppressor protein p53 induces apoptosis by transactivating the expression of numerous pro-apoptotic genes, including *Apaf-1, Asc, Bax, Bid, Caspase-6, Fas, Fas-L, Noxa, p21* $^{Cip1/WAF1}$, and *Puma* (Miyashita and Reed, 1995;

Muller *et al.*, 1998; Maecker *et al.*, 2000; Sax *et al.*, 2002; Crow *et al.*, 2004). In fact, in addition to cell cycle regulation, $p21^{Cip1/WAF1}$ has been shown to have pro-apoptotic activities as disruption of $p21^{Cip1/WAF1}$ expression decreased cell death (Sheikh *et al.*, 1995; Yang *et al.*, 2001). It was previously shown that CLA-mix could induce p53-dependent and –independent apoptosis in different cell types (Belury, 2002; Majumder *et al.*, 2002). Moreover, the 10E, 12Z-CLA but not the 9Z, 11E-CLA was able to increase the expression of $p21^{WAF1/CIP1}$ gene in prostate cancer PC-3 cells (Ochoa *et al.*, 2004). Here, we demonstrated that CLA-mix and its isomer, 9E, 11E-CLA, might induce apoptosis of WEHI-3B JCS cells in a p53-dependent manner, since the expressions of both *p53* and *p21* genes were markedly increased in CLA-mix- and 9E, 11E-CLA-treated WEHI-3B JCS cells as shown in chapter 3.

Based on the results so far obtained, a proposed mechanism by which CLA-mix and the 9E, 11E-CLA might exert their pro-apoptotic activity on the WEHI-3B JCS cells is described as follows. During the activation of "intrinsic" pathway, they down-regulate the anti-apoptotic genes of the Bcl-2 family, such as *Bcl-$_{XL}$*, and up-regulate the pro-apoptotic genes, including *Bak* and *Bad*, to form MPT which are responsible for the loss of $\Delta\Psi m$ and subsequent mitochondrial release of cyt c into the cytosol. cyt c binds to Apaf-1 and recruits procaspase-9 to form an apoptosome. This leads to autocatalytic activation of procaspase-9 to caspase-9. Activated caspase-9 then cleaves and activates the procaspase-3 into activated caspase-3. Moreover, CLA-mix and the 9E, 11E-CLA isomer also up-regulate the gene expression of *p53* and *p21*, which have pro-apoptotic activities and might be responsible for some of the apoptotic events in the "intrinsic" pathway. In fact, our results are in agreement with a recent report showing that CLA-mix induces apoptosis in human breast cancer MDA-MB-231 cells through the "intrinsic" pathway by up-regulating the protein

expression of Bak, down-regulating the protein expression of Bcl-$_{XL}$, inducing the translocation of cyt c from the mitochondria to the cytosol, and promoting the cleavage of procaspases-3 and -9 (Miglietta *et al.*, 2005).

On the other hand, our results also demonstrated that CLA-mix and the 9E, 11E-CLA isomer can trigger apoptosis through the "extrinsic" pathway. They up-regulate the protein expression of the death receptor Fas and its agonist Fas-L. This cell death process can be enhanced by wild-type p53, which up-regulates *Fas* expression at the transcriptional level (Nagata, 1999; Crow *et al.*, 2004). Various tumor cells such as leukemia, hepatoblastoma, neuroblastoma, and brain tumor cells are killed by cytotoxic drugs or γ-irradiation in a Fas-dependent manner. When Fas is activated, the adaptor molecule FADD is recruited and binds to Fas, which is responsible for recruiting procaspase-8 by its DED. As a result, the procaspase-8 is activated to caspase-8 which then cleaves and activates caspases-3. Our findings also showed that CLA-mix and the 9E, 11E-CLA isomer can activate caspases-8 and -3.

Therefore, CLA-mix and the 9E, 11E-CLA isomer can trigger apoptosis through both the "intrinsic" and "extrinsic" pathways in the murine myelomonocytic leukemia WEHI-3B JCS cells. Since both the "intrinsic" and "extrinsic" pathways are entwined, this contributes to an amplification of cyt c release which may give profound therapeutic implications: when drugs targeted to both pathways are combined, this cocktail therapy may contribute a synergistic effect for induction of apoptosis. In addition to these pathways, CLA-mix and the 9E, 11E-CLA might also trigger apoptosis through an oxidative stress mediated by the ROS-dependent cell death pathway. Nevertheless, whether the loss of membrane integrity is a direct result of MPT formed by pro-apoptotic Bcl-2 family members or due to ROS reacting with membrane lipids remains to be elucidated.

# CHAPTER 5

# STUDIES ON THE DIFFERENTIATION-INDUCING ACTIVITY OF CONJUGATED LINOLEIC ACID ON MYELOID LEUKEMIA CELLS

# 5.1 Introduction

During the last two decades, differentiation therapy of cancer has been a subject of intense investigation (Spira and Carducci, 2003; Zelent *et al.*, 2005). Acute promyelocytic leukemia (APL) is the first model of hematologic malignancies treated by differentiation-inducing agents including the well-known inducer, all-*trans*-retinoic acid (ATRA) (Fang *et al.*, 2002). Very often, differentiation therapy is used with or following cycles of low-dose conventional chemotherapy. It has been shown that combination of cytotoxic and differentiation therapies can enhance tumor cell apoptosis, reduce recurrence of malignancies and alleviate development of drug resistance (Lo *et al.*, 2002). One of the characteristics of myeloid leukemia is the neoplastic proliferation of hematopoietic progenitor cells which lose the capability to differentiate. Differentiation therapy is thereby an alternative approach for the treatment of myeloid leukemia in which the immature leukemia cells are induced to attain a mature phenotype when exposed to differentiation inducers (Hozumi, 1994; Leszczyniecka *et al.*, 2001) and terminal differentiation of leukemia cells is usually accompanied by the cessation of cell proliferation (Leszczyniecka *et al.*, 2001). Table 5.1 summarizes the common differentiation inducers employed in clinical trials for hematologic malignancies.

It had been reported previously that various CLA isomers, including the *cis*-9, *trans*-11 CLA, *cis*-9, *cis*-11 CLA, *trans*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA, induced monocytic differentiation of the human promyelocytic leukemia HL60 cells, as assessed by their ability to induce expression of the LPS receptor CD14, on the leukemic cell surface (Yu *et al.*, 2002). Since this is the only documentation to date narrating the differentiation-inducing activity of CLA, yet its action mechanisms to

trigger differentiation of myeloid leukemia cells remain poorly understood. In the present study, a mixture of CLA isomers (CLA-mix) was investigated for its effects on the differentiation of the murine myelomonocytic leukemia WEHI-3B JCS cells in terms of morphological, phenotypic, and functional changes. Morphological changes in the CLA-treated WEHI-3B JCS cells were studied by staining of cytocentrifuged preparations. The changes in size and granularity were also analyzed by flow cytometry. Additionally, phenotypic changes in the WEHI-3B JCS cells following CLA-mix administration were studied by determining the expression of certain cell surface markers of monocytic (Mac-1 and F4/80) and granulocytic (Gr-1) differentiation. Furthermore, other differentiation-associated functional characteristics such as monocytic serine esterase (MSE) and endocytic activities of WEHI-3B JCS cells subsequent to CLA-mix exposure were also studied.

Since previous work in our laboratory had shown that hematopoietic cytokines such as tumor necrosis factor-α (TNF-α) (Mak *et al.*, 1993; Leung *et al.*, 1994), and interleukin-1 (IL-1) (Chan *et al.*, 1997) may play a role in triggering the monocytic differentiation of the murine myeloid leukemia WEHI-3B JCS cells, therefore, the modulatory effect of CLA-mix on the expression of these cytokine genes in the leukemia WEHI-3B JCS cells was also examined.

**Table 5.1: Common differentiation inducers used in clinical trials for hematologic malignancies.**

| **Differentiation inducers** | **Examples** |
|---|---|
| Anti-proliferative agents | Interferons;<br>Arsenic trioxide ($As_2O_3$);<br>Chemotherapeutic agents - cytosine arabinoside; hydroxyurea |
| Cyclic AMP analogs | 8-chloro-cyclic-cAMP (8-Cl-cAMP) |
| Demethylating agents | Aza-cytidine |
| Histone deacetylase inhibitors (HDACi) | Suberoylanilide hydroxamic acid (SAHA);<br>Depsipeptide;<br>Sodium phenylbutyrate (NaPB) |
| Hematopoietic cytokines | Erythropoietin (EPO);<br>Granulocyte colony-stimulating factor (G-CSF);<br>Granulocyte-macrophage colony-stimulating factor (GM-CSF);<br>Interleukin-3 (IL-3);<br>Interleukin-6 (IL-6) |
| Lipids | Alkylophospholipids;<br>Gangliosides;<br>Short-chain fatty acids;<br>Sodium butyrate |
| PKC agonists and antagonists | Bryostatin;<br>Tetradecanoylphorbol acetate (TPA) |
| Polar-planar compounds | Hexamethylene bisacetamide (HMBA) |
| Vitamin analogs | Retinoic acid and its derivatives;<br>Vitamin $D_3$ |

(Modified from Miller and Waxman, 2002)

# 5.2 Results

## 5.2.1 Morphological Alterations in CLA-mix- and CLA isomer-treated WEHI-3B JCS Cells

Murine myelomonocytic leukemia WEHI-3B JCS cells were examined for their morphological changes following treatment with CLA-mix. Briefly, WEHI-3B JCS cells were cultured with CLA-mix (25-200 μM) at 37°C for 48 hours. The cells were then cytocentrifuged onto microscopic slides and stained with Hemacolor solutions. As shown in Fig. 5.1, CLA-mix induced the morphological differentiation of the WEHI-3B JCS cells in a dose-dependent manner. The differentiated cells (Fig. 5.1 B-F) had typical macrophage-like morphology as there was an increase in the cytoplasm to nucleus ratio as well as increase in vacuolation in the CLA-treated cells when compared with the control cells (Fig. 5.1 A). Differential counting of the cytospin preparations showed that ~44% of WEHI-3B JCS cells cultured with 150 μM of CLA for 48 hours had acquired the characteristics of mature macrophages (Table 5.2). Additionally, we also examined the morphological changes of the WEHI-3B JCS cells after exposure to various CLA isomers. It is interesting to find out that CLA isomers, including *cis*-9, *trans*-10 CLA (Fig. 5.2), *trans*-10, *cis*-12 CLA (Fig. 5.3), and *trans*-9, *trans*-11 CLA (Fig. 5.4), could induce morphological changes of WEHI-3B JCS cells with characteristics of mature macrophages. However, the *cis*-9, *cis*-11 CLA did not exhibit any differentiation-inducing activity on WEHI-3B JCS cells (data not shown). Therefore, the results suggest that some CLA isomers can induce differentiation of WEHI-3B JCS cells along the monocytic lineage.

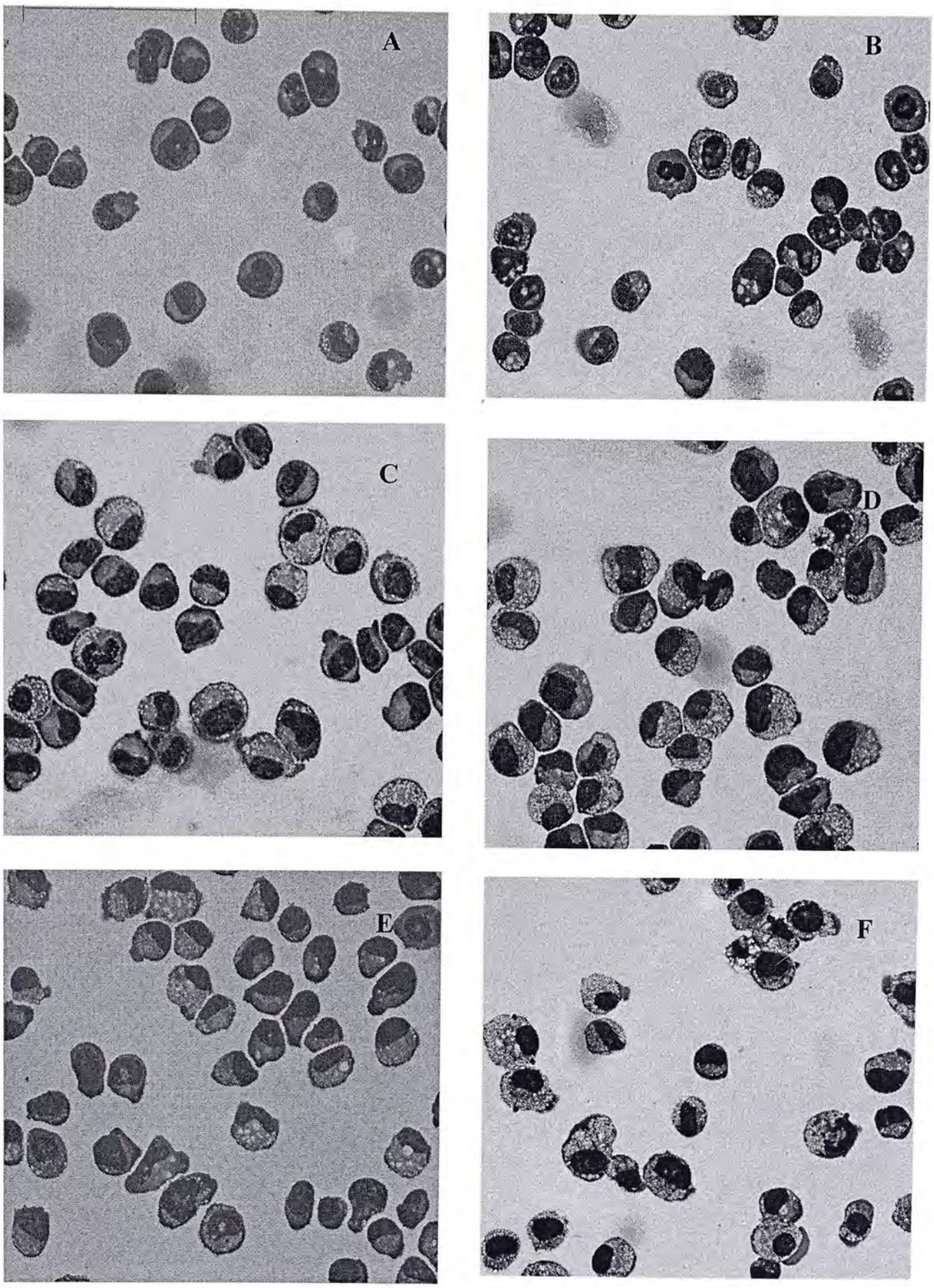



**Fig. 5.1: Morphological changes in CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) solvent control (0.1% ethanol); (B) 25 μM CLA-mix; (C) 50 μM CLA-mix; (D) 100 μM CLA-mix; (E) 150 μM CLA-mix; or (F) 200 μM CLA-mix at 37°C for 48 hours. The cells were cytocentrifuged onto microscopic slides and were then stained with Hemacolor solutions (Magnification x 200).

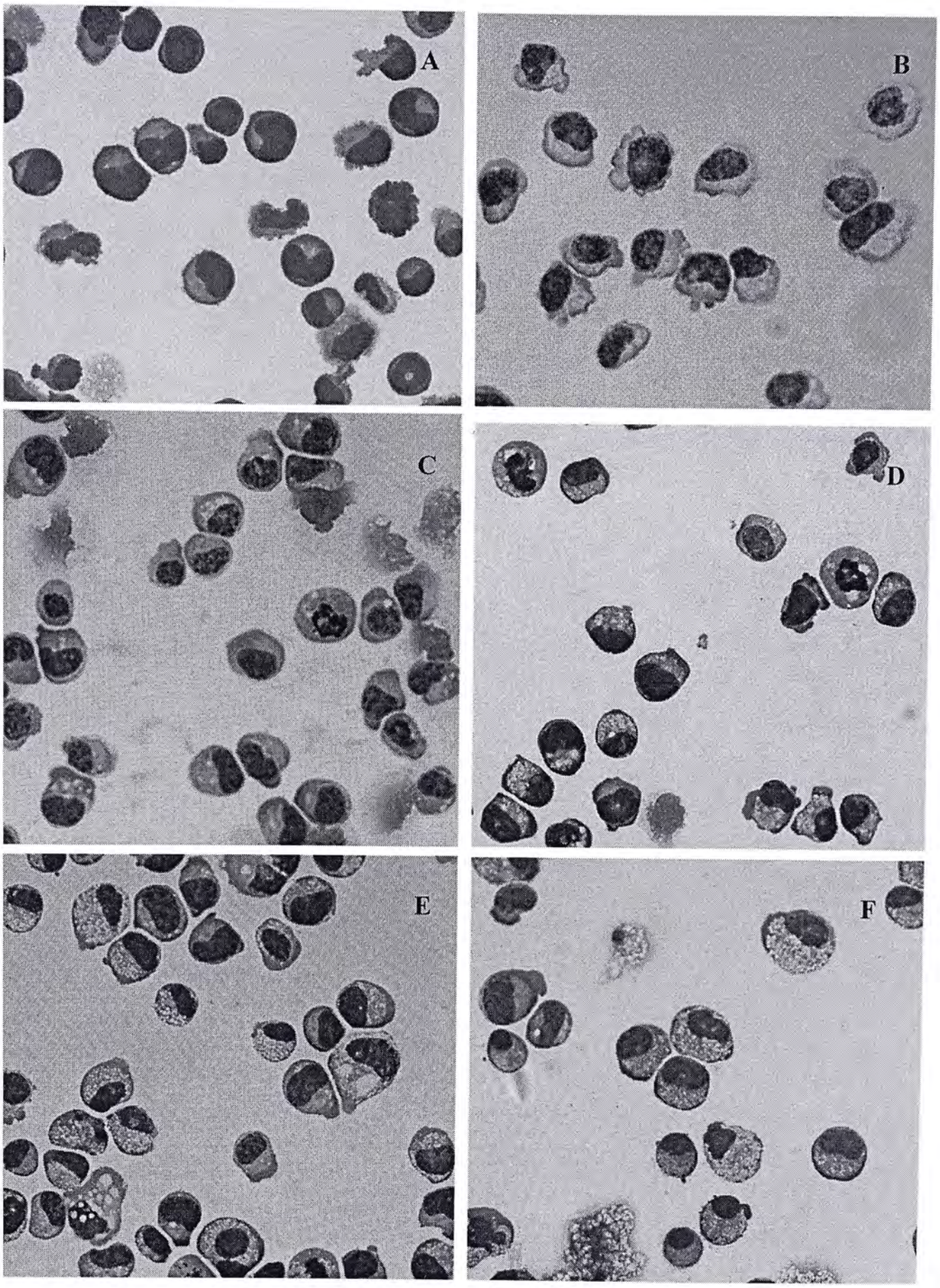



**Fig. 5.2: Morphological changes in *cis*-9, *trans*-11 (9Z, 11E) CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) solvent control (0.1% ethanol); (B) 25 μM 9Z, 11E-CLA; (C) 50 μM 9Z, 11E-CLA; (D) 100 μM 9Z, 11E-CLA; (E) 150 μM 9Z, 11E-CLA; or (F) 200 μM 9Z, 11E-CLA at 37°C for 48 hours. The cells were cytocentrifuged onto microscopic slides and were then stained with Hemacolor solutions (Magnification x 200).

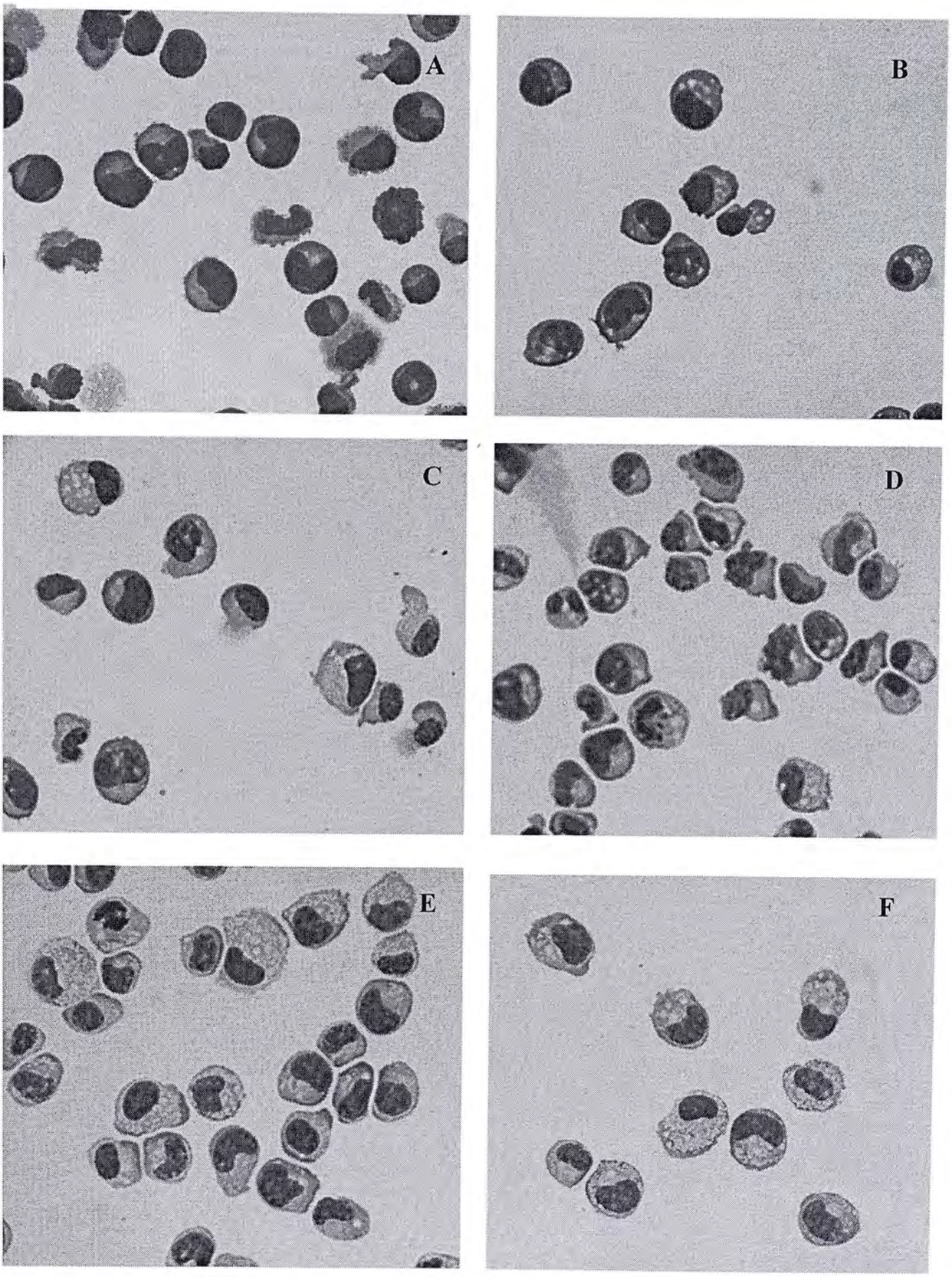



**Fig. 5.3: Morphological changes in *trans*-10, *cis*-12 (10E, 12Z) CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) solvent control (0.1% ethanol); (B) 25 μM 10E, 12Z-CLA; (C) 50 μM 10E, 12Z-CLA; (D) 100 μM 10E, 12Z-CLA; (E) 150 μM 10E, 12Z-CLA; or (F) 200 μM 10E, 12Z-CLA at 37°C for 48 hours. The cells were cytocentrifuged onto microscopic slides and were then stained with Hemacolor solutions (Magnification x 200).

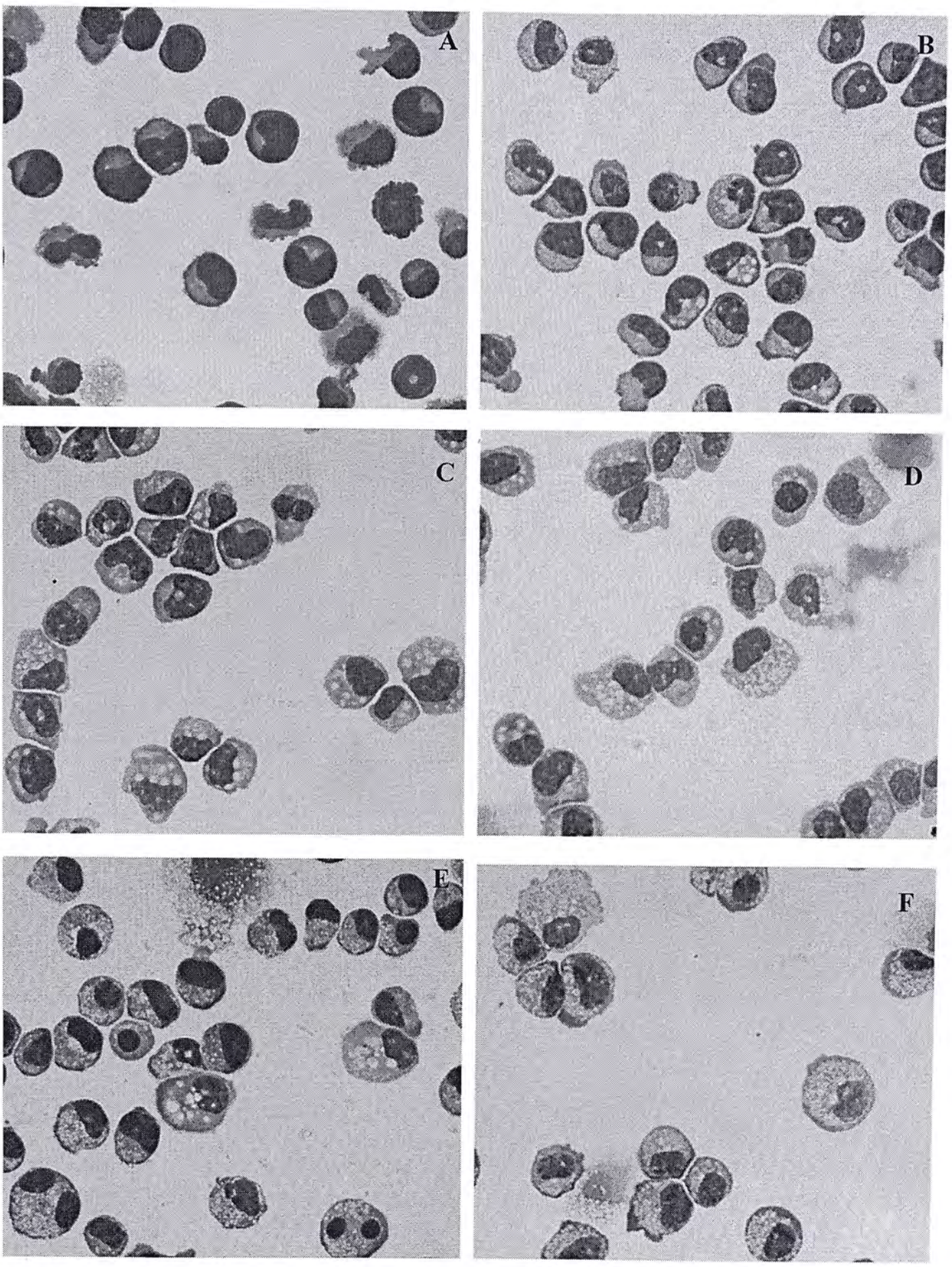



**Fig. 5.4: Morphological changes in *trans*-9, *trans*-11 (9E, 11E) CLA-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) solvent control (0.1% ethanol); (B) 25 μM 9E, 11E-CLA; (C) 50 μM 9E, 11E-CLA; (D) 100 μM 9E, 11E-CLA; (E) 150 μM 9E, 11E-CLA; or (F) 200 μM 9E, 11E-CLA at 37°C for 48 hours. The cells were cytocentrifuged onto microscopic slides and were then stained with Hemacolor solutions (Magnification x 200).

**Table 5.2: Morphological differentiation of CLA-treated WEHI-3B JCS cells.**

| Treatment of cells with | Percentage of different cell types (mean ± SD) | | |
|---|---|---|---|
| | Immature blast cells | Intermediate stages cells | Mature macrophage -like cells |
| Solvent control | 89.7 ± 2.4 | 10.3 ± 2.4 | 0 |
| CLA (25 μM) | 48.8 ± 5.3 | 41.2 ± 2.4 | 10.0 ± 2.9 |
| CLA (50 μM) | 12.5 ± 0 | 60.8 ± 6.7 | 26.7 ± 2.7 |
| CLA (150 μM) | 11.2 ± 1.3 | 44.7 ± 0.6 | 44.1 ± 1.8 |

WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with solvent control or various concentrations of CLA (25, 50, and 150 μM) at $37^0$C for 48 hours. Cytospin preparations were made and cells were stained with Hemacolor staining set for morphological identification of cells. The stained cells were scored as the immature blast cells (myeloblasts), intermediate stages cells (myelocytes and promonocyte-like cells) and mature macrophage-like cells. At least 300 cells were scored for each determination.

### 5.2.2 Effects of CLA-mix on the Cell Size and Granularity of WEHI-3B JCS Cells

To further analyze the differentiation-inducing activity of CLA-mix, the changes in cell size and granularity in CLA-mix-treated WEHI-3B JCS cells were studied by flow cytometry. Forward scatter (FSC) indicates the cell size and side scatter (SSC) indicates the internal complexity of the cells. As illustrated in Fig. 5.5, CLA-mix significantly increased the SSC of the WEHI-3B JCS cells. Such increase could also be demonstrated in the SSC of the human promyelocytic leukemia NB4 cells treated with CLA-mix (data not shown). Therefore, these results further support that the granularity of the CLA-treated WEHI-3B JCS cells had increased as a result of cellular differentiation.

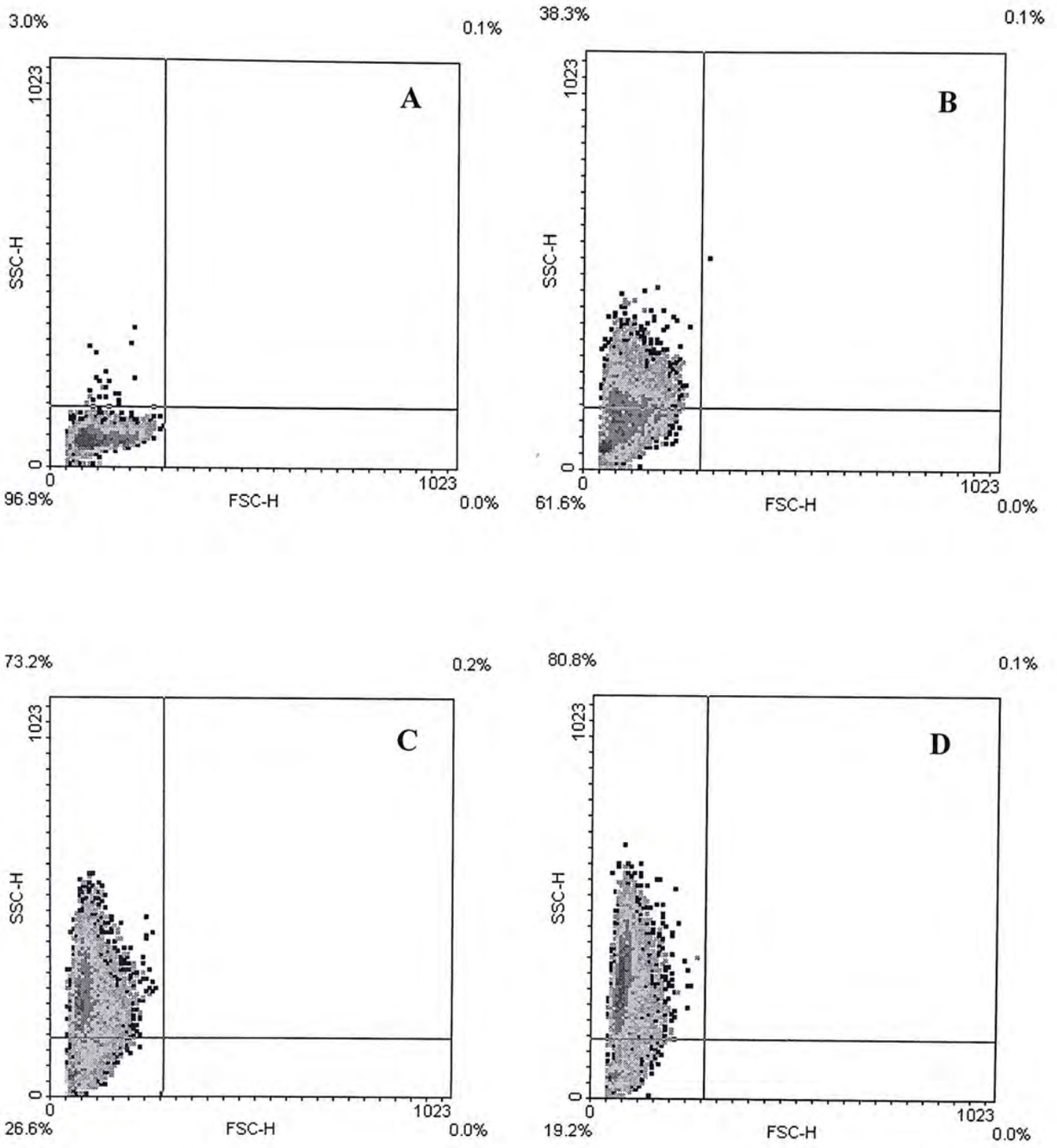



**Fig. 5.5: Effects of CLA-mix on the cell size and granularity of murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) solvent control (0.1% ethanol); (B) 50 μM CLA-mix; (C) 100 μM CLA-mix; or (D) 200 μM CLA-mix at 37°C for 48 hours. The paraformaldehyde-fixed cells were analyzed for FSC and SSC using the FACSort flow cytometer.

### 5.2.3 Studies of the Surface Phenotypic Changes in the CLA-mix-treated WEHI-3B JCS cells

Cellular differentiation is usually accompanied by phenotypic changes on the cell surface of differentiated cells, as determined by the expression of certain lineage-specific differentiation antigens. They can be thereby used as markers for studying cell differentiation. Previous work had demonstrated that enhanced expressions of Mac-1 and F4/80 surface markers were seen in WEHI-3B JCS cells undergoing monocytic differentiation (Mak *et al.* 1993; Chan *et al.*, 1997). As shown in Fig. 5.6, the expression of macrophage differentiation antigens Mac-1 and F4/80 in WEHI-3B JCS cells was significantly augmented dose-dependently upon exposure to CLA-mix. However, the expression of granulocytic marker Gr-1 was similar in both control and CLA-mix-treated cells. These results suggest that CLA-mix triggers differentiation of WEHI-3B JCS cells along the monocytic pathway with the acquisition of macrophage-like phenotypes.

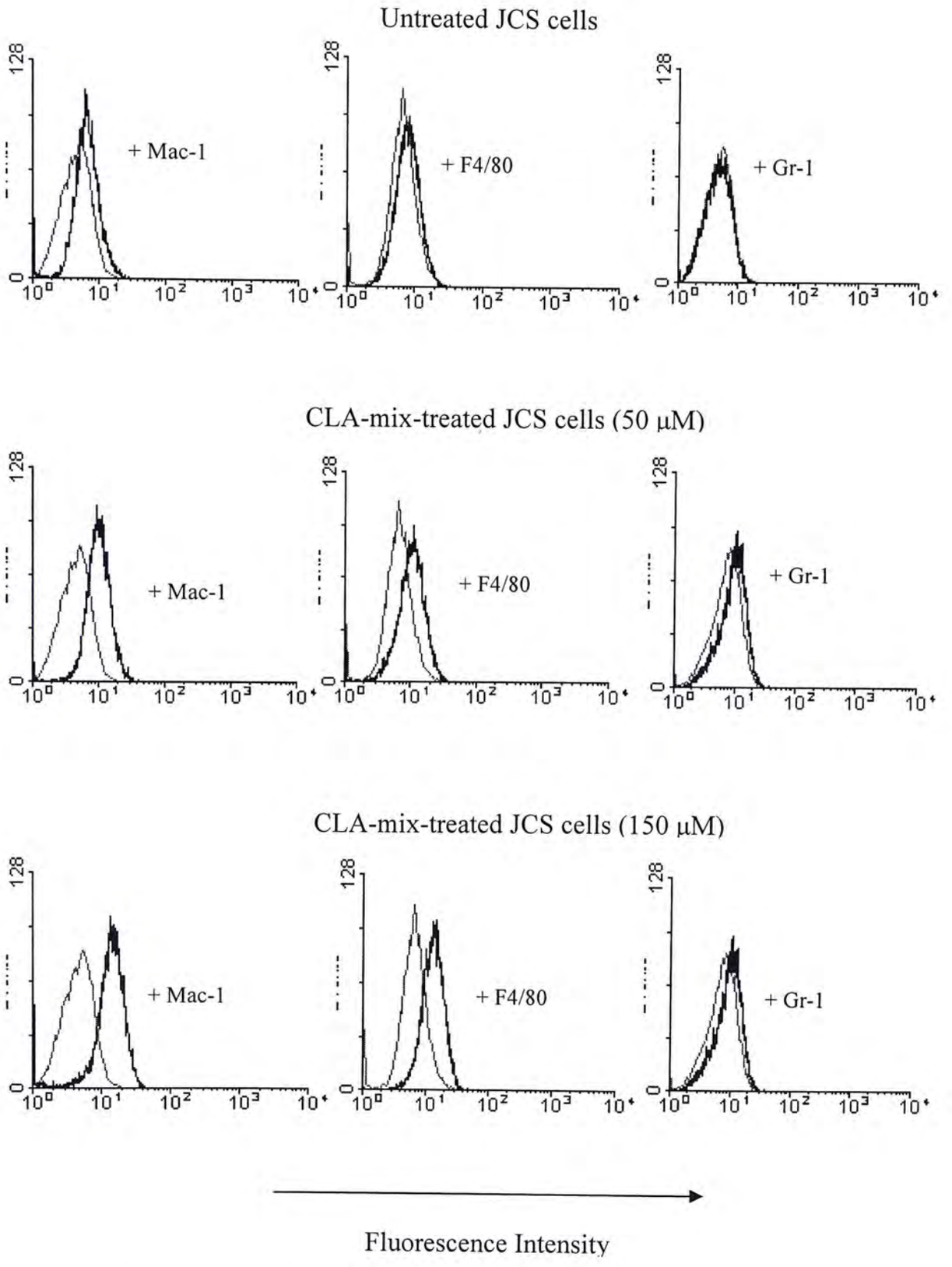



**Fig. 5.6: Studies of the surface phenotypic changes in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by flow cytometry.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with CLA-mix (0, 50 and 150 μM) at 37°C for 48 hours. After harvesting, cells were stained with rat monoclonal antibodies to Mac-1, F4/80 and Gr-1 antigen respectively. Control cells were stained with isotype-matched antibodies.

### 5.2.4 Studies on the Induction of Monocytic Serine Esterase (MSE) Activity in the CLA-mix-treated WEHI-3B JCS Cells

Monocytic Serine Esterase (MSE), usually referred as the non-specific esterase, is a cytoplasmic marker for monocytic differentiated cells (Yam *et al.*, 1971). MSE converts fluorescein diacetate into a fluorescent product which is then measured by flow cytometry. Activity of MSE can be primarily detected in monocytes, macrophages and histocytes. Therefore the assessment on activity of MSE is exclusive to cells of monocytic but not granulocytic lineage. As shown in Fig. 5.7, the MSE activity of WEHI-3B JCS cells was increased significantly in a dose-dependent manner by exposure to CLA-mix.

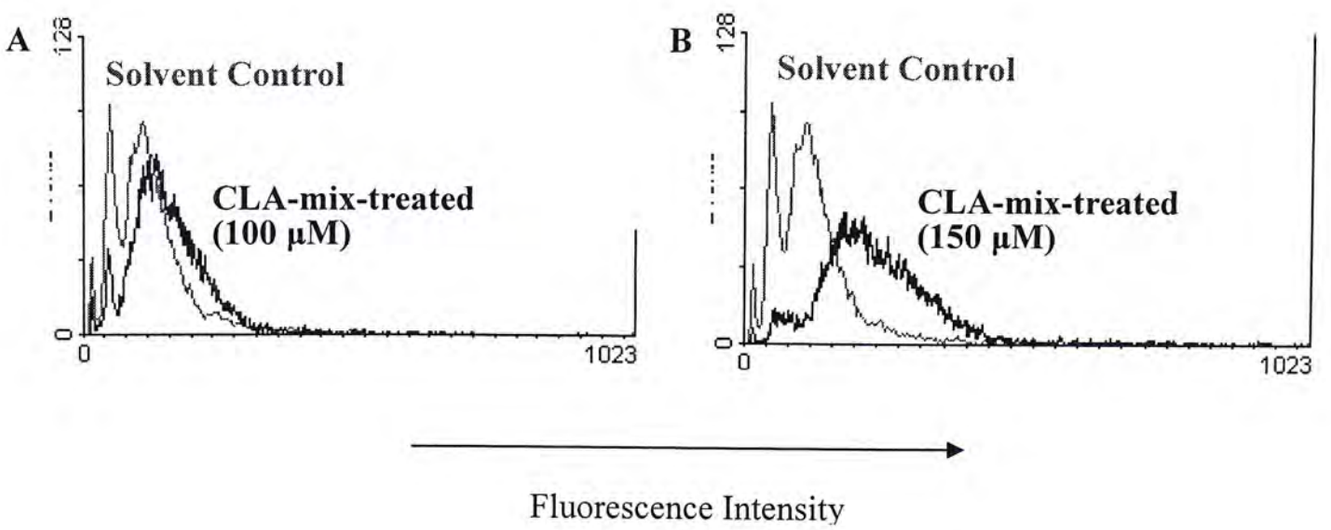



**Fig. 5.7: Studies on the induction of monocytic serine esterase activity in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by flow cytometry.** WEHI-3B JCS cells ($10^4$ cells/ml) were incubated with (A) 100 μM CLA-mix; or (B) 150 μM CLA-mix at 37°C for 3 days. Cells were harvested and then incubated with fluorescein diacetate. The fluorescence intensity was determined by the FACSort flow cytometer.

### 5.2.5 Studies on the Induction of Endocytic Activity in the CLA-mix-treated WEHI-3B JCS Cells

It has been reported previously that monocytic differentiation of WEHI-3B JCS cells is always accompanied by increased endocytic activity (Leung *et al.*, 1994). In this study, the endocytic activity was determined by flow cytometric analysis on the uptake of fluorescent FITC-conjugated bovine serum albumin (FITC-BSA). As illustrated in Fig. 5.8, the endocytic activity of the CLA-mix-treated WEHI-3B JCS cells was increased dose-dependently after 3-day treatment with CLA-mix.

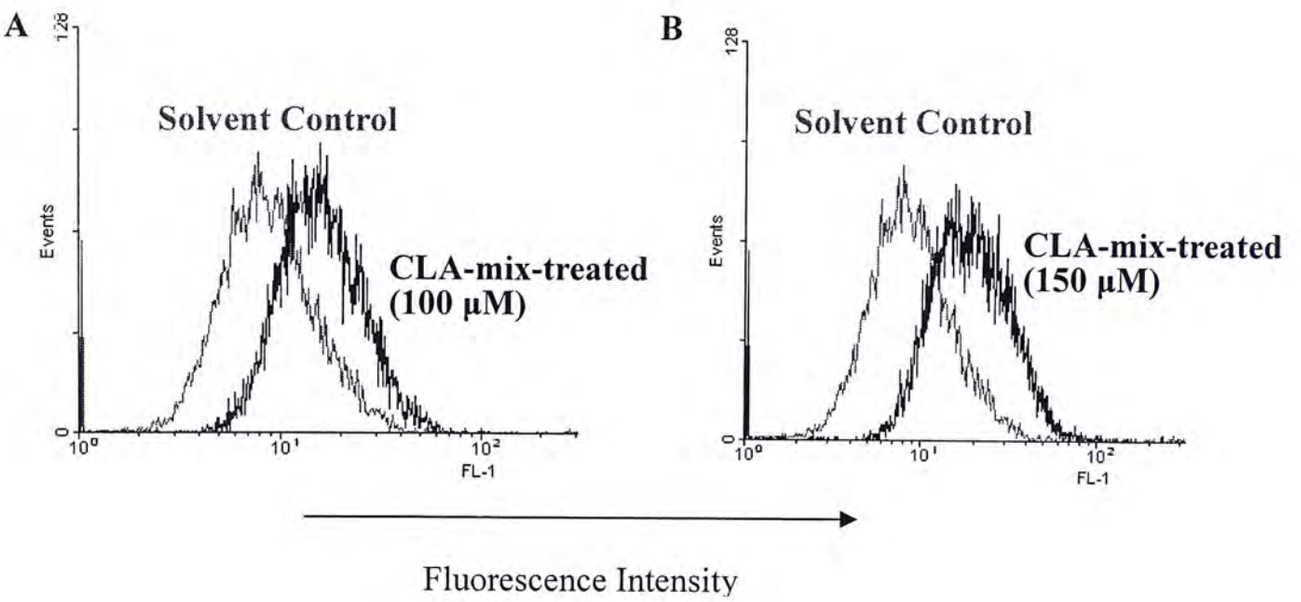



**Fig. 5.8: Studies on the induction of endocytic activity in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells by flow cytometry.** WEHI-3B JCS cells ($10^4$ cells/ml) were pre-treated with (A) 100 µM; or (B) 150 µM CLA-mix at 37°C for 3 days and then incubated with FITC-conjugated BSA at 37°C for 6 hours. The fluorescence intensity was determined by the FACSort flow cytometer.

### 5.2.6 Studies on the Expression of the Differentiation-regulatory Cytokine Genes in the CLA-mix-treated WEHI-3B JCS Cells

In addition to chemical inducers, several hematopoietic cytokines are also known to promote the differentiation of various leukemia cell lines along lineage-restricted pathways as revealed in Table 5.1. For examples, as reported in a recent review article (Tsiftsoglou *et al.*, 2003), granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-1, and IL-6 were found to induce differentiation of murine myeloblastic leukemia M1 cells towards the macrophage phenotype in the years 1988 and 1989. In 1990, transforming growth factor-β (TGF-β) had been documented to promote differentiation of the human monoblast-like lymphoma U-937 cells into macrophages. In addition, leukemia inhibitory factor (LIF) and tumor necrosis factor-α (TNF-α) were found to promote macrophage development in M1 and WEHI-3B JCS cells as reported in 1991 and 1993, respectively. Furthermore, in 1997, erythropoietin (EPO) and thrombopoietin (TPO) were found to induce differentiation of human megakaryoblastic leukemia UT-7/GM cells into erythrocytic and megakaryocytic phenotype, respectively.

Therefore, in this study, the technique of RT-PCR was employed to analyze the expression of certain cytokine genes, such as TNF-α, IL-1β, and IFN-γ, in the WEHI-3B JCS cells after treatment with 150 μM CLA-mix for different time intervals. As shown in Fig. 5.9, the expression of TNF-α and IFN-γ genes was significantly increased at 3-hour after treatment with CLA-mix, which peaked at the 12 hour and then declined thereafter. On the other hand, the expression of IL-1β gene was also enhanced at 3-hour after treatment with CLA, but continued to increase in a time-dependent manner and the level remained high at the 24 hour after CLA treatment.

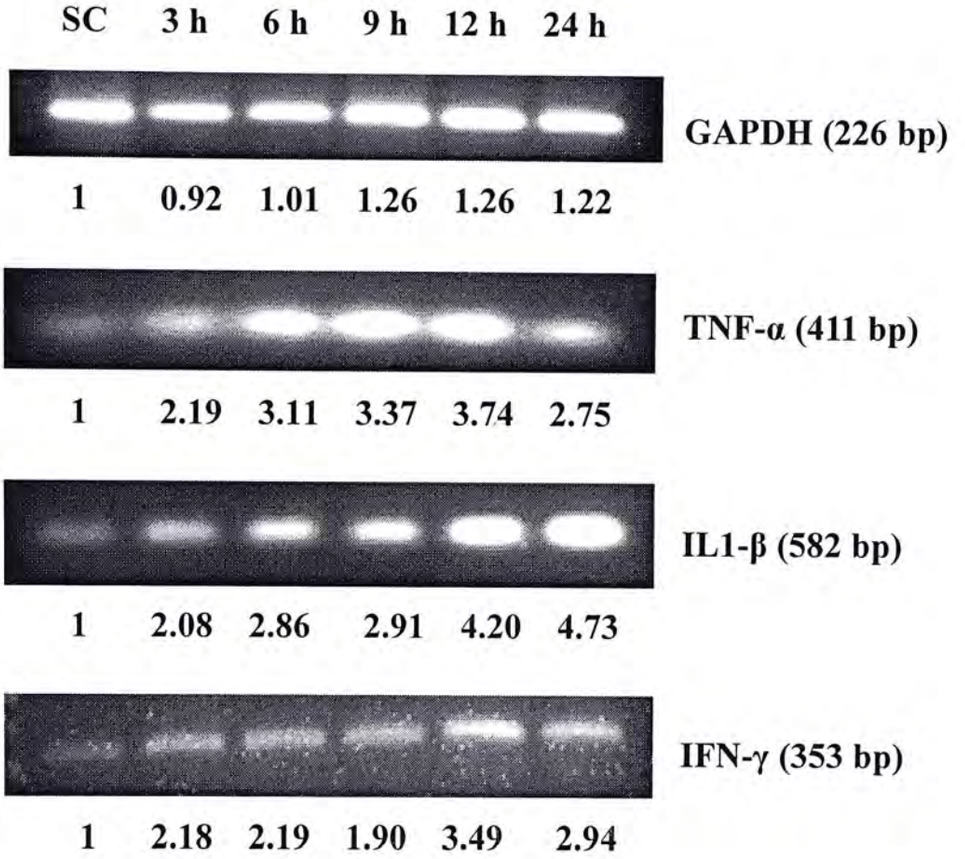



**Fig. 5.9: Expression of the differentiation-regulatory cytokine genes in the CLA-mix-treated murine myelomonocytic leukemia WEHI-3B JCS cells.** WEHI-3B JCS cells ($10^6$ cells) were incubated with 150 μM CLA-mix at 37°C for different time intervals (3, 6, 9, 12 and 24 hours) or treated with ethanol (SC) as a control. Total RNA were extracted by TRIZOL reagent with the method described in Chapter 2. The RNA were reverse transcribed and amplified by PCR using specific primer pairs. The number of amplification cycles was 22 for GAPDH, 28 for TNF-α and IL-1β and 24 for IFN-γ respectively. The PCR products were then separated on an ethidium bromide-stained agarose gel (2%). The amount of PCR products was quantified by ImageQuant. The value at the bottom of each band represents the relative intensity after normalization with respect to GAPDH, and comparison was made with the corresponding solvent control.

## 5.3 Discussion

Normally hematopoietic stem cells (HSC) are programmed to differentiate along restricted cell lineage pathways during blood cell development through regulation of certain external growth factors that maintain the balance of cell proliferation, differentiation, and apoptosis in the bone marrow (Tsiftsoglou *et al.*, 2003). Accumulating evidence indicates that leukemia cells fail to differentiate leading to accumulation of immature blast cells in the bone marrow (Warner *et al.*, 2004). Therefore, differentiation therapy has become a novel approach for the treatment of cancer including hematological malignancies (Leszczyniecka *et al.*, 2001) as commitment of leukemia cells into differentiation pathways is accompanied by irreversible maturation and growth inhibition. Previous reports in our laboratory have documented that a subclone (JCS) of the murine myelomonocytic leukemia WEHI-3B cells could be induced to differentiate along the monocytic lineage by cytokines such as TNF-α (Mak *et al.*, 1993; Leung *et al.*, 1994) and IL-1 (Chan *et al.*, 1997) and isoflavones such as biochanin A (Fung *et al.*, 1997). The leukemia WEHI-3B JCS cell line is a good *in vitro* model for studying the growth and differentiation of myeloid leukemia cells as the WEHI-3B cells retain the capacity to undergo monocytic or granulocytic differentiation in response to various biological or chemical agents (Mak et al., 1993; Gamba-Vitalo et al., 1986).

In this study, the differentiation-inducing activity of a mixture of CLA isomers in the murine myelomonocytic leukemia WEHI-3B JCS cells was demonstrated. Present findings showed that non-cytotoxic concentrations of CLA-mix (50-200 μM) could induce terminal differentiation of WEHI-3B JCS cells into cells with characteristics of macrophages, as shown by morphological, phenotypic and functional studies. In terms

of morphology, CLA-mix increased the cytoplasm/nucleus ratio and vacuolation of WEHI-3B JCS cells. Interestingly, CLA isomers including the *cis*-9, *trans*-11 CLA, the *trans*-10, *cis*-12 CLA, and the *trans*-9, *trans*-11 CLA had also found to induce morphological changes of the WEHI-3B JCS cells with characteristics of mature macrophages. Therefore the results suggest that all these three CLA isomers can induce monocytic differentiation of WEHI-3B JCS cells. In addition, flow cytometric analysis on the forward scatter (FSC) and side scatter (SSC) of the WEHI-3B JCS cells also demonstrated that the SSC was markedly increased following CLA-mix treatment. This further supports that the granularity of the CLA-mix-treated WEHI-3B JCS cells was increased as a result of cellular differentiation. In terms of phenotype and functions, the increased expression of macrophage differentiation antigens (Mac-1 and F4/80) but not the granulocyte differentiation antigen (Gr-1), in addition to induction of monocytic serine esterase and endocytic activities by CLA-mix are evidence for WEHI-3B JCS cells undergoing monocytic rather than granulocytic differentiation. Present findings are in line with a recent report which showed that various CLA isomers could induce the monocytic differentiation of the human promyelocytic leukemia HL-60 cells, as measured by the increased expression of the cell surface marker CD14 (Yu *et al.*, 2002). Interestingly, the differentiation-inducing activity of CLA isomers was also reported for cultured human preadipocytes (McNeel *et al.*, 2003), however, when the mouse clonally-derived 3T3-L1 preadipocyte cell line was used, CLA was found to inhibit their differentiation (Brodie *et al.*, 1999; Kang *et al.*, 2003). These results suggest that the ability of CLA to modulate preadipocyte differentiation may depend on whether primary or cloned cell lines are used or there may be species differences.

The molecular mechanisms by which CLA-mix can induce differentiation of

myeloid leukemia cells remain elusive. It is well known that several hematopoietic cytokines (such as TNF-α, IL-1β and IFN-γ) are effective in inducing lineage-restricted myeloid leukemic cell differentiation (Olsson *et al.*, 1996). Our study also showed that TNF-α, IL-1 and IFN-γ genes were up-regulated in WEHI-3B JCS cells as early as 3 hours and their levels remained high up to 24 hours after exposure to CLA-mix. It is possible that the increased expression of these cytokine genes may be related with WEHI-3B JCS cell maturation and acquisition of macrophage characteristics. Similarly, the ability of CLA to increase the IL-2 and IFN-γ cytokine gene expression was also reported in the Jurket T leukemia cell line (Luongo *et al.*, 2003). Nevertheless, the underlying mechanisms for CLA-mix-induced monocytic differentiation of myeloid leukemia cells require further investigations. Recently, it has been demonstrated that activators of peroxisome proliferator-activated receptor γ (PPARγ) are protective against cancers of the colon, prostate, and mammary gland (Sporn *et al.*, 2001). The PPARγ is a ligand-activated nuclear transcription factor belonging to the steroid receptor superfamily (Chang and Szabo, 2000). Whereas much is known about the role of PPARγ in adipocytic differentiation (Yu *et al.*, 2002), its activation also leads to anti-neoplastic activity by inducing many cancer cell lines, including bladder cancer (Guan *et al.*, 1999), breast cancer (Mueller *et al.*, 1998), and non-small cell lung cancer (Chang and Szabo, 2000), to undergo differentiation and to revert their immature malignant phenotype back to more mature, less malignant phenotype (His *et al*, 2002; Maggiora *et al.*, 2004). In fact, PPARγ is highly expressed in adipose tissue, adrenal gland, colon, mammary gland, prostate, and macrophages (Belury, 2002; Yu *et al.*, 2002). Interestingly, the induction of PPARγ was correlated with the anti-proliferative effect of CLA treatment (Maggiora *et al.*, 2004). Since CLA shares many functional similarities with ligands of PPARγ (Yu *et al.*, 2002), CLA is thought to activate the

responsive genes of PPARγ by forming Δ6-desaturated products (Belury, 2002; Yu *et al.*, 2002). Many groups have also reported the involvement of PPARγ in macrophage differentiation (Tontonoz *et al.*, 1998) as well as in regulating macrophage gene expression (Ricote *et al.*, 2000). Nevertheless, the mechanisms of myeloid differentiation involving PPARγ remain controversial as studies using PPARγ-deficient stem cells showed that PPARγ is not essential for macrophage differentiation, both *in vitro* and *in vivo* (Moore *et al.*, 2001). In addition, it has also been reported that PPARγ plays a role specifically in lipogenesis but not in the differentiation of human promyelocytic leukemia NB4 cells (Inazawa *et al.*, 2003). The discrepancy of data between different cell models suggests that the role of the activated PPARγ in CLA-induced myeloid leukemic cell differentiation awaits further investigations. More studies should be done to elucidate the molecular mechanisms and the signaling pathways by which CLA can trigger myeloid leukemic cell differentiation. In conclusion, since CLA could trigger leukemic cell differentiation and ultimately contribute to proliferative quiescence, therefore, our findings suggest that CLA or its isomers might have therapeutic potentials in the differentiation therapy against hematologic malignancies, particularly the myeloid leukemia.

# CHAPTER 6

# CONCLUSIONS AND FUTURE PERSPECTIVES

Conjugated linoleic acid (CLA) comprises a family of positional and geometric isomers of linoleic acid (LA). The major dietary sources of CLA are derived from ruminants, for instance, it can be found in many dairy products. The predominant isomer of CLA, the *cis*-9, *trans*-11 CLA (9Z, 11E-CLA), can be produced directly by bacterial biohydrogenation in the rumen or by Δ9-desaturation of the vaccenic acid (*trans*-11) in most mammalian tissues including humans. However, the yield from the latter process does not contribute significantly to any beneficial effect on our health. The second most copious isomer of CLA is the *trans*-10, *cis*-12 CLA (10E, 12Z-CLA), which has been identified in grilled beef as a potential anti-carcinogen (Wahle *et al.*, 2004). Numerous health benefits of a mixture of CLA isomers (CLA-mix) have been mainly attributed to the 10E, 12Z-CLA isomer. Interestingly, recent studies revealed that these two predominant isomers, in fact, act through different signaling pathways to exhibit diverse physiological and pharmacological activities, as demonstrated in a variety of animal models, to assuage conditions such as obesity, atherosclerosis, carcinogenesis, tumor formation, and to delay the onset of diabetes and enhance our immune system (Belury, 2002). Among all these bioactivities, the anti-tumor activities of CLA have received much attention in recent years. CLA has been found to suppress tumor growth and serve as a cytostatic or cytotoxic mediator to various human cancer cell lines.

As shown in Chapter 3, CLA-mix could inhibit the proliferation of murine myelomonocytic leukemia WEHI-3B JCS cells in a dose- and time-dependent manner. Similar growth-inhibitory effect of CLA-mix was also demonstrated in other murine and human leukemia cell lines including M1, HL-60, NB4, and K-562, as well as in the human lymphoma U-937 cells. Moreover, CLA-mix showed the most potent anti-proliferative activity on WEHI-3B JCS cells when compared to its parental fatty

acid, LA, and the four isomers being investigated. Among all the isomers tested, the *trans*-9, *trans*-11 CLA (9E, 11E-CLA), the 10E, 12Z-CLA and the 9Z, 11E-CLA were found to have similar anti-proliferative activity on the leukemia WEHI-3B JCS cells. Interestingly, our findings showed that the all-*trans* CLA (9E, 11E-CLA) was more potent than the all-*cis* CLA (9Z, 11Z-CLA) to inhibit the proliferation of WEHI-3B JCS cells. Therefore, more studies should be done to elucidate the structure-function relationship of different CLA isomers. Previous studies had demonstrated that the major effects of CLA are largely observed in mice treating with a mixture of CLA isomers or the 10E, 12Z-CLA but not the 9Z, 11E-CLA (Pariza *et al*, 2001; Wahle *et al.*, 2004). It is more likely that the differential effects of different CLA isomers are due to their different structures. For instance, since 9Z, 11E-CLA and linoleic acid are similar in structure, it is possible that the 9Z, 11E-CLA isomer might alter arachidonic acid metabolism so as to modulate carcinogenesis (Pariza *et al.*, 2001; Ochoa *et al.*, 2004). On the other hand, the 10E, 12Z-CLA isomer has been shown to induce apoptosis in a variety of tumor cell lines (Palombo *et al.*, 2002; Yamasaki *et al.*, 2002; Oh *et al.*, 2003; Maggiora *et al.*, 2004; Ochoa *et al.*, 2004; Kim *et al.*, 2005). Our results also indicate that interactions might occur among individual CLA isomers as the anti-proliferative activity of CLA-mix is greater than each of its four isomers. Nevertheless, the observed anti-proliferative activity of CLA-mix was most likely to be due to its cytostatic but not cytotoxic effect on WEHI-3B JCS cells as assessed by the trypan blue exclusion assay. Moreover, our results also indicated that CLA-mix exhibited minimal cytotoxic effect on normal cells such as the murine peritoneal macrophages.

In order to elucidate the mechanisms by which CLA and its isomers could trigger their anti-proliferative activity on the WEHI-3B JCS cells, their effects on the cell

cycle, apoptosis and differentiation of WEHI-3B JCS cells were investigated. Our findings revealed that CLA-mix, the 10E, 12Z-CLA, and the 9E, 11E-CLA could trigger cell cycle arrest at the $G_0$ / $G_1$ phase. To date, this is the first report demonstrated the "extraordinarily" anti-proliferative activity of the 9E, 11E-CLA isomer. Therefore, it was chosen for further mechanistic investigations. Using the technique of RT-PCR, both CLA-mix and the 9E, 11E-CLA were found to modulate the expression of several cell cycle-regulatory genes. The mRNA levels of the *p53* gene were increased following exposure to CLA-mix and the 9E, 11E-CLA. In addition, both CLA-mix and the 9E, 11E-CLA up-regulated the gene expression of the cyclin-dependent kinase inhibitor (CKI) $p21^{CIP1/WAF1}$ , while the expression of *cyclin A* gene was down-regulated. Moreover, CLA-mix also increased the mRNA levels of another CKI, the $p27^{KIP1}$, which is known to inhibit cell cycle progression from $G_1$ to S phase. Interestingly, we are the first to show that the 9E, 11E-CLA isomer could activate the $G_1$ checkpoint of the leukemia cells by modulating cell cycle-regulatory genes including *p53*, $p21^{CIP1/WAF1}$, and *cyclin A*. As both CLA-mix and the 9E, 11E-CLA had been found to induce the gene expressions of *p53* and $p21^{CIP1/WAF1}$, this suggests that CLA induces $G_0$ / $G_1$ phase cell cycle arrest in a p53-dependent manner.

Since CLA was found to exhibit inhibitory effects on the leukemia WEHI-3B JCS cell growth and cell cycle transition at the $G_1$ phase, the suppressive effect of CLA on the tumorigenicity of the WEHI-3B JCS cells in syngeneic BALB/c mice was also examined. Our results showed that pretreatment of WEHI-3B JCS cells *in vitro* with either the CLA-mix or the 9E, 11E-CLA isomer could significantly reduce the *in vivo* growth of the leukemia cells in a dose-dependent manner. Nonetheless, the preponderance of the anti-tumor researches of CLA is the *in vitro* studies. Therefore,

more studies should be carried out using *in vivo* tumor models to demonstrate the anti-tumor activity of CLA, which is a necessary step before putting CLA into clinical trials.

The induction of cell cycle arrest could be one of the possible mechanisms leading to the observed anti-proliferative activity of CLA on myeloid leukemia cells. Other possible mechanisms include the induction of apoptosis and differentiation of myeloid leukemia cells. Our results showed that CLA-mix and two of the CLA isomers, 10E, 12Z-CLA and 9E, 11E-CLA, were able to trigger apoptosis in WEHI-3B JCS cells while the other two CLA isomers, 9Z, 11E-CLA and 9Z, 11Z-CLA, failed to do so. In fact, this is the first report documented that the 9E, 11E-CLA isomer exerts the most potent pro-apoptotic activity on WEHI-3B JCS cells when compared with other CLA isomers being investigated. Our findings also showed that both CLA-mix and the 9E, 11E-CLA isomer could induce mitochondrial membrane depolarization in the WEHI-3B JCS cells, suggesting that the intrinsic pathway may be involved in the CLA-induced apoptosis of the WEHI-3B JCS cells.

From the pro-apoptotic gene expression studies of CLA-mix- or 9E, 11E-CLA-treated WEHI-3B JCS cells, it was found that the anti-apoptotic *Bcl-x*$_L$ gene was down-regulated while the pro-apoptotic *Bak* and *Bad* genes were up-regulated. Therefore, our results suggest that CLA triggers apoptosis of WEHI-3B JCS cells through transcriptional activation of the pro-apoptotic genes of the Bcl-2 family. Moreover, we had also examined the translational regulation of certain apoptosis-regulated proteins by CLA. Our results showed that the protein expression of the death receptor Fas and its ligand, Fas-L, were both up-regulated in the CLA-mix- and 9E, 11E-CLA-treated WEHI-3B JCS cells. These present findings

suggest that apoptosis of the WEHI-3B JCS cells triggered by CLA-mix and the 9E, 11E-CLA isomer signals through both the "intrinsic" and "extrinsic" pathways. This was further confirmed by studies on the activities of caspases-3, -8 and -9. It was found that both CLA-mix and the 9E, 11E-CLA isomer increased significantly the activities of "initiator" caspases-8 and -9 as well as "effector" caspase-3 in the WEHI-3B JCS cells.

To sum up, a proposed mechanism by which CLA-mix and the 9E, 11E-CLA might exert their pro-apoptotic activity on the WEHI-3B JCS cells is described as follows. During the "intrinsic" pathway, they down-regulate the anti-apoptotic genes of the Bcl-2 family, such as *Bcl-$_{XL}$*, and up-regulate the pro-apoptotic genes, including *Bak* and *Bad*, to form mitochondrial membrane permeability transition (MPT) which are responsible for the loss of mitochondrial potential ($\Delta\Psi m$) and subsequent mitochondrial release of cytochrome c (cyt c) into the cytosol. Nevertheless, whether CLA-mix and the 9E, 11E-CLA isomer can induce the translocation of cyt. from mitochondria into cytosol awaits further investigation. Cyt c binds to an adaptor protein, Apaf-1, and recruits procaspase-9 to form an apoptosome. This leads to autocatalytic activation of procaspase-9 to caspase-9. Activated caspase-9 is an "initiator" caspase that cleaves and activates the effector caspases such as caspase-3. Moreover, as discussed before, CLA-mix and the 9E, 11E-CLA isomer also up-regulate the gene expression of *p53* and *p21*, which also have pro-apoptotic activities and might be responsible for some of the apoptotic events in the "intrinsic" pathway.

In addition, the results also demonstrated that CLA-mix and the 9E, 11E-CLA isomer can trigger apoptosis through the "extrinsic" pathway. When Fas is activated,

the adaptor molecule FADD is recruited and binds to Fas, which is responsible for recruiting procaspase-8 by its DED. As a result, the procaspase-8 is activated to caspase-8 which is also an "initiator" caspase and can cleave and activate caspase-3.

In addition to these two apoptotic pathways, CLA-mix and the 9E, 11E-CLA might also trigger apoptosis through an oxidative stress mediated by ROS-dependent cell death pathway. In our study, we found that CLA-mix and the 9E, 11E-CLA isomer enhanced the superoxide anion ($O_2^-$) production in the WEHI-3B JCS cells. Interestingly, prior treatment of WEHI-3B JCS cells with antioxidants such as SOD or NAC significantly suppressed the production of $O_2^-$ and partly blocked apoptosis in both CLA-mix-treated and 9E, 11E-CLA-treated WEHI-3B JCS cells. As a result, it could be speculated that over-production of ROS in CLA-treated WEHI-3B JCS cells may result in detrimental damages to the cell such as lipid peroxidation, DNA adduct formation, protein oxidation, and enzyme inactivation, and all these events can ultimately lead to the death of the leukemia cells. However, it is still uncertain whether the loss of membrane integrity in CLA-induced apoptosis of leukemia cells is a direct result of MPT formed by pro-apoptotic Bcl-2 family members or reactions of reactive oxygen species such as the $O_2^-$ with membrane lipids. Nevertheless, whether CLA-mix and the 9E, 11E-CLA isomer can trigger apoptosis through other apoptotic pathways such as the endoplasmic reticulum stress-induced apoptotic pathway and caspase-independent apoptotic pathway is an intriguing aspect that is worthy of future investigation (Vermeulen et al., 2005).

Apart from the anti-proliferative and apoptosis-inducing activities of CLA, we also demonstrated that CLA isomers could exhibit differentiation-inducing activity in the murine myelomonocytic leukemia WEHI-3B JCS cells. It has been reported that

the WEHI-3B JCS cells can retain the capacity to undergo monocytic or granulocytic differentiation in response to certain signals (Mak *et al.*, 1993; Leung *et al*, 1994; Chan *et al.*, 1997; Fung *et al.*, 1997). Present findings showed that CLA-mix and its isomers could induce terminal differentiation of WEHI-3B JCS cells into cells with characteristics of mature macrophages, as judged by a number of morphological, phenotypic and functional criteria.

In terms of morphology, CLA-mix increased the cytoplasm/nucleus ratio and vacuolation of WEHI-3B JCS cells. CLA isomers including the 9Z, 11E-CLA, the 10E, 12Z-CLA, and the 9E, 11E-CLA had also found to induce morphological changes of the WEHI-3B JCS cells in a dose-dependent manner, and the induced cells had the characteristics of mature macrophages. We therefore believe that all these CLA isomers can induce monocytic differentiation of WEHI-3B JCS cells. In addition, flow cytometric analysis on the forward scatter (FSC) and side scatter (SSC) of the WEHI-3B JCS cells also demonstrated that the SSC was markedly increased following CLA-mix administration, indicating that an increase in granularity in leukemia cell is associated with cellular differentiation. In terms of phenotype and functions, the increase in expression of macrophage differentiation antigens, Mac-1 and F4/80, but not the granulocyte differentiation antigen, Gr-1, as well as the induction of monocytic serine esterase (MSE) and endocytic activities by CLA-mix are evidence to support that CLA-mix induces monocytic rather than granulocytic differentiation in WEHI-3B JCS cells.

The molecular mechanisms by which CLA-mix can induce differentiation of myeloid leukemia cells remain largely elusive. It is well known that several hematopoietic cytokines, such as TNF-$\alpha$, IL-1 and IFN-$\gamma$, are effective in inducing

lineage-restricted myeloid leukemia cell differentiation (Olsson *et al.*, 1996). Our findings also showed that the expression of TNF-α, IL-1β and IFN-γ genes was upregulated in WEHI-3B JCS cells upon exposure to CLA-mix. It is possible that the increased expression of these cytokine genes may be related with WEHI-3B JCS cell maturation and acquisition of macrophage characteristics. Nevertheless, the underlying mechanisms for CLA-mix-induced monocytic differentiation of myeloid leukemia cells await further investigations.

In conclusion, since CLA and some of the CLA isomers can inhibit the growth of myeloid leukemia WEHI-3B JCS cells *in vitro*, possibly through triggering $G_0$ / $G_1$ phase cell cycle arrest, inducing apoptosis and leukemic cell differentiation, CLA and its isomers might have therapeutic potentials against the hematologic malignancies, particularly the myeloid leukemia. Interestingly, CLA shares many functional similarities with ligands of PPARγ (Yu *et al.*, 2002), and the anti-proliferative activity of CLA treatment was correlated with the induction of PPARγ (Maggiora *et al.*, 2004). Moreover, Miglietta and coworkers (2005) have recently reported that CLA induces apoptosis through reduction of the ERK/MAPK signaling (Miglietta *et al.*, 2005). Nonetheless, the signaling pathways by which CLA exerts anti-tumor activities on myeloid leukemia cells have not been fully elucidated. There are still many gaps in our knowledge on the action mechanisms by which CLA inhibits the growth of myeloid leukemia cells.

Despite all the promising findings, definitive evidence on the safety and efficacy of CLA as a "drug" in the treatment of myeloid leukemia in humans are still lacking. It is obvious that more in-depth studies are required to elucidate the anti-tumor activity of CLA *in vivo*. By unraveling the molecular mechanisms and signaling

pathways by which CLA can modulate the proliferation, apoptosis, and differentiation of myeloid leukemia cells, it is hoped that novel treatment for some forms of myeloid leukemia can be developed with higher efficacy and minimal toxicity in the near future.

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