CHAPTER I

INTRODUCTION

1.1 Statement of problem

Cancer is a group of diseases characterized by uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and detectable cancer. Cancer is treated with surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy.

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All cancers caused by cigarette smoking and heavy use of alcohol could be prevented completely. The American Cancer Society (ACS) estimates that in 2008 about 170,000 cancer deaths are expected to be caused by tobacco use. Scientific evidence suggests that about one-third of the 565,650 cancer deaths expected to occur in 2008 will be related to overweight or obesity, physical inactivity, and nutrition and

thus could also be prevented. Certain cancers are related to infectious agents, such as hepatitis B virus (HBV), human papillomavirus (HPV), human immunodeficiency virus (HIV), *Helicobacter pylori (H. pylori)*, and others, and could be prevented through behavioural changes, vaccines, or antibiotics. In addition, many of the more than 1 million of skin cancers that are expected to be diagnosed in 2008 could have been prevented by protection from the sun's rays and avoiding indoor tanning (1).

The National Institutes of Health (NIH) estimate overall costs of cancer in 2007 at $219.2 billion: $89.0 billion for direct medical costs (total of all health expenditures); $18.2 billion for indirect morbidity costs (cost of lost productivity due to illness); and $112.0 billion for indirect mortality costs (cost of lost productivity due to premature death). Lack of health insurance and other barriers prevent many Americans from receiving optimal health care. According to early release estimates from the 2006 National Health Interview Survey (NHIS), about 24% of Americans aged 18-64 and 13% of children had no health insurance coverage for at least part of the past year. Almost 34% of adults who lack a high school diploma were uninsured in the past year, compared to 23% of high school graduates and 15% of those with more than a high school education (1).

According to a report from the Bureau of Health Policy and Planning, Ministry of Public Health of Thailand, cancer is the leading cause of death. In 2004, the cancer death rate was 81.3 per 100,000 populations. The cancer death rate has been gradually increasing year, form 58.6 in 1999 to 81.3 in 2004 (2). Moreover, cancer was major cause of death in Chiang Mai province in the years 2002 and 2003 (3).

Leukemia is a group of cancer involving the blood-forming organs, and is characterized by uncontrolled increase of white blood cells. An estimated 259,889

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people in the United States are living with, or are in remission from, leukemia. An estimated 43,050 new cases of leukemia will be diagnosed in the United States in 2010. Chronic leukemias account for 11 percent more cases than acute leukemias. Most cases occur in older adults; the median patient age at diagnosis is 66 years. Leukemia is expected to strike more than 10 times as many adults as children in 2010 (About 39,733 adults compared with 3,317 children, aged 0-14 years). About 31 percent of cancers in children aged 0-14 years are leukemia. The most common cancer in children 1 to 7 years old is acute lymphocytic leukemia (ALL) which will account for about 5,330 new cases in 2010 (4).. The most common types of leukemia in adults are acute myelogenous leukemia (AML), with an estimated 12,330 new cases in 2010, and chronic lymphocytic leukemia (CLL), with about 14,990 new cases in 2010. Chronic myelogenous leukemia (CML) is estimated to affect about 4,870 persons in 2010. Development of leukemia has been linked to certain environmental and genetic risk factors, including exposure to radiation, toxic chemicals, chemotherapeutic agents, viral and other microorganisms, genetic disorders associated with chromosomal instability, cigarette smoking and other environmental factors. These factors lead to genetic changes leukemic cells that in turn affect the functions of other genes, including tumor suppressor genes and oncogenes. Some cases of mutation in oncogenes have provided useful biological markers for monitoring the course of the disease during treatment. The detection of overexpression in specific oncogenes or tumor suppressor genes provides information that is useful in the diagnosis of leukemia and prognosis of the disease. Thus, the *Wilms' tumor 1* (*WT1*) gene overexpression is a good biological maker for diagnosis and treatment monitor leukemias (5).

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Interestingly, Anuchapreeda *et al.* (2006) showed that curcumin affected the cell viability and proliferation in K562 cell line (6). It also showed the inhibitory effects on *WT1* gene expression in leukemia patient's cells and four leukemic cell lines (K562, Molt4, HL-60, and U937) (6-8). Furthermore, curcumin was also shown to induce cell death in two leukemic cells, K562 and Jurkat cells (9) and demonstrated that the dietary component of curcumin at 3.5 µg/mL, the low concentration, induces apoptosis in human leukemia HL-60 cells (10). Up until now, the inhibitory mechanism of pure curcumin on *WT1* gene expression has been remained unclear.

Turmeric, which is also known as curcumin (*Curcuma longa* Linn) is one of the most popular herbs used for medical treatment, due to its wide variety of medicinal properties such as anti-inflammatory, antibacterial, antifungal, antioxidant, anticarcinogen, antimutagen, and anticancer properties. The active compounds of turmeric are known as curcuminoids, yellow pigmented substances isolated from the rhizome of turmeric. This active extract can be subdivided into three distinct components: pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcuminoids are a group of diet-derived agents that are being clinically evaluated as chemopreventive agents for major cancer targets, including breast, prostate, lung, stomach, duodenal, colon cancers, and leukemias (11, 12). Curcuminoids, especially pure curcumin, have strong anticancer agent and anti-tumor progression properties (13-15).

The *WT1* gene encodes 48-57 kDa protein. There are four zinc fingers which act as a transcriptional activator or repressor. This protein will bind to DNA target sequences, depending on the cellular or chromosomal context. The *WT1* gene is involved in growth regulation and is among a group of genes that are necessary for

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the induction of cell differentiation (16-18), such as platelet-derived growth factor A (PDGF-A) chain (19), *c-myc*, *bcl-2* (20), colony-stimulating factor-1 (CSF-1) (21), transforming growth factor-β1 (TGF-β1) (22), insulin-like growth factor 1 receptor (IGF1R) (23), insulin-like growth factor II (24), retinoic acid receptor-α (RAR-α) and RNA metabolism (25-27). Thus, it is critical for development of many organs in enthe gonads, kidneys, liver, spleen, heart, and mesenchymal structure (28-30). Somatic homozygous mutation or loss of WT1 predisposes for the pediatric kidney cancer Wilms' tumor (16, 17). In normal hematopoietic cells, WT1 is expressed at high levels in a subset of progenitors and is found at low levels in mature cells (31-37). In addition, many study observations support the notion of WT1 as a potential oncogene in the development of human leukemia. *WT1* gene is highly expressed in most leukemias (5, 38-40) and its expression is further increased upon relapse of the disease (41).

The *WT1* gene is located on the human chromosome 11q13. In addition, four different isoforms of WT1 which resulted from the combination of two alternative splices have been reported in WT1-expressing cells (41). Alternative splice I insert 17 amino acids (+17AA) in exon 5 and alternative splice II insert 3 amino acids (+KTS) in exon 9 which exists between zinc-fingers 3 and 4, and yields four isoforms. There are WT1 17AA(+) KTS(+) or WT1 +/+, 17AA(+)KTS(-) or WT1 +/-, 17AA(-)KTS(+) or WT1 -/+, and 17AA(-)KTS(-) or WT1 -/-, each of which is considered to have different functions. All of the four isoforms were expressed in primary human solid cancers, including lung cancer (42), head and neck squamous cell carcinoma (HNSCC) (43), sarcoma (44), and human primary leukemia (45). Among the four WT1 isoforms, WT1+/+ isoform was dominantly expressed in all of

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the cancers examined. The results showing that constitutive expression of WT1 +/+ isoform rescued the growth inhibitory effect of WT1 antisense oligomers on cancer cells (46) indicated the contribution of WT1 +/+ isoform to the growth of cancer cells (47). The functions of WT1 +/- isoform involve in blocking differentiation of T lymphoid progenitor cells and in the tumorigenesis of lymphoid malignancy (48). Moreover, it also increased the expression levels of *Bcl-2* gene in G401 rhabdoid cells (49), indicating that the *WT1* gene might be involved in suppression of cell apoptosis. The WT1 -/+ isoform involve in inducing G1 arrest in osteosacroma cell line whereas the WT1 -/- isoform modulates cytoskeletal dynamics (50). It inhibited G1/S progression and accelerated differentiation in 32D cl3 murine myeloid progenitor in response to G-CSF (51). Furthermore, *WT1* gene expression was found to involve in the promotion of cell cycle, as was indicated by findings that suppression of WT1 expression induced G2/M or G1 block in human leukemia K562 cells and HER2/neu overexpressing breast cancer cells, respectively (52, 53). These results indicate that each of the WT1 isoforms has different functions and play important roles in leukemogenesis and tumorigenesis.

The goals of this study are to investigate the inhibitory mechanism of pure curcumin on *WT1* gene expression and WT1 protein activity in K562 cells and investigate the effect of pure curcumin on post-translation modification of exogenous WT1 +/+ protein in transfected U937 cells. These goals are based on the finding that the decreasing of *WT1* gene expression by pure curcumin might be related to a degradation pathway or intracellular signaling pathway.

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1.2 Literature review

1.2.1 Leukemia

Leukemia (American English) or leukaemia (British English; Greek leukos *λευκός*, "white"; aima *αίμα*, "blood") is a cancer of the blood or bone marrow characterized by an abnormal increase of blood cells, usually leukocytes (white blood cells). Leukemia is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases called hematological neoplasms.

1.2.1.1 Etiology and risk factor

The origin of leukemia at the genetic level in most cases appears to be related to mutation and altered expression of oncogenes and tumor suppressor genes. Most oncogenes regulated cell proliferation and differentiation. Abnormal oncogene or tumor suppressor gene expression induced by translocation and genetic fusion or mutation often results in unregulated cellular proliferation. Although the events that lead to this are not entirely understood, a number of host and environmental factors have been identified that are associated with increased risk of leukemic transformation.

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1.2.1.2 Host factor

1.2.1.2.1 Hereditary defect in leukemia

Leukemia does not appear to be inherited, although some individuals have an increased predisposition for acquiring it. There is an increased incidence of leukemia in family members of leukemic patients. An identical twin of a patient with acute leukemia possesses a markedly increased relative risk of developing leukemia. These

finding do not rule out shared environmental factors; in fact, recent molecular data have shown that the shared risk in twins may be owing to shared placental circulation (and possible in utero exposure) rather than to an inherited genetic mutation.

1.2.1.2.2 Congenital chromosomal abnormalities

Leukemia occurs with increased frequency in patients with congenital disorders that have an inherited tendency for chromosomal fragility (example, Bloom's syndrome and Fanconi's anemia) or with an abnormal chromosome constitution (example, Downs' syndrome, Klinefelter's syndrome and Turner's syndrome). A 10-20 fold increased incidence of acute leukemia is seen in children with Down syndrome. This risk may be partially explained by the discovery that the *AML1* gene, associated with some cases of AML, has been identified on chromosome 21 in a region believed to be responsible for the Down syndrome phenotype.

1.2.1.2.3 Immunodeficiency

An unusually high incidence of lymphoproliferative disease (lymphoid leukemia and lymphoma) has been noted in patients with hereditary immunodeficiency states, such as ataxia-tekeangiectasia and six-linked agammaglobulinemia.

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1.2.1.2.4 Chronic marrow dysfunction

Patient with chronic marrow dysfunction syndrome have an increased risk of acute leukemic transformation. Examples include the myelodysplastic syndrome,

myeloproliferative disorder, aplastic anemia, and paroxysmal nocturnal hemoglobinuria.

1.2.1.3 Environmental factor

1.2.1.3.1 Ionizing radiation

Leukemia is associated with exposure to ionizing radiation; this fact is dramatically illustrated in data collected in population exposed to the use of nuclear weapons in Hiroshima and Nagasaki. The occurrence of leukemia in this population is many times that of individuals not exposed to ionizing radiation, and it is highest in those survivors with the greatest exposure. Both acute and chronic forms to leukemia were reported, including AML, ALL, and CML. The role of exposure to electromagenetic radiation in causation of leukemia is controversial.

1.2.1.3.2 Chemicals and drugs

Many chemicals and drugs have been associated with development of leukemia. In humans, benzene is the most frequently include chloramphenicol and phenylbutazone. Certain cytotoxic chemotherapeutic agents, especially alkylating drugs, are also associated with leukemic transformation, and the risk in patients receiving alkylating agents is increased by use of therapeutic radiation. This has been noted particularly in patients treated with combined chemoradiotherapy for Hodgkin's disease. Secondary AML cases related to prior treatment with epipodophyllotoxins and other topoisomerase inhibitors are often associated with 11q23 abnormalities and a poor prognosis (54).

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Smoking has been implicated as a risk factor (albeit slight) for AML in adults. Results of studies on maternal smoking have been inconclusive in childhood acute leukemias. Some studies have shown increased risk of childhood AML with maternal alcohol consumption during pregnancy.

1.2.1.3.3 Viruses

The human T-cell leukemia/lymphoma virus-1 (HTLV-1) has been implicated as the causative agent of adult T-cell leukemia/lymphoma (ATL). This rare form of leukemia has a mature helper-inducer T-cell phenotype. ATL is endemic to south-western Japan, the Caribbean basin, Africa, the south-eastern United States, and several other geographic regions (55). Another related virus, HTLV-2, has been isolated from patients with atypical hairy-cell leukemia (a chronic lymphoid leukemia). The Epstein-Barr virus has been linked to malignancies, including Africa burkitt's lymphoma, a high-grade B-cell lymphoma that may have leukemic manifestation (56).

1.2.1.4 Incidence

Recently, the American Cancer Society reported that 44,270 new cases are expected in 2008, with slightly more cases of chronic (19,940) than acute (18,720) disease. Leukemia is diagnosed 10 times more often in adults than in children, although it is often thought of as primarily a childhood disease. Acute lymphocytic leukemia accounts for approximately 72% (3,040/4,220) of the leukemia cases among children (ages 0-19 years). In adults, the most common types are acute myeloid leukemia and chronic lymphocytic leukemia. The incidence of acute myeloid leukemia increased by an average of 2.1% per year from 1988 to 2001, but decreased sharply by about

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6% per year from 2001-2004. In contrast, the incidence of chronic lymphocytic leukemia has decreased gradually by less than 1% per year since 1990. Deaths: an estimated 21,710 deaths are expected to occur in 2008. Death rates in males and females combined have decreased by about 0.8% per year since 1995.

1.2.1.5 Leukemia classification

The two most commonly used classification schemata for leukemia, are the older French-American-British (FAB) system and the newer World Health Organization (WHO) system.

In 1976, FAB cooperative group classified AML and ALL into subcategories on the basis of morphological and cytochemical characteristics. Further, the classification has undergone several modifications which, together with cytogenetics, immunological markers, and *in vitro* cell culture findings, may provide a more accurate prognostic, therapeutic, and investigational subclassification of the acute leukemias (57-59). According to this classification, AML was classified into 8 subcategories (M0 to M7) while ALL was classified into 3 subcategories, L1 to L3 (Table 1).

In 1986, the first Morphologic, Immunologic, and Cytogenetic (MIC) Cooperative study group published the criteria for classification of ALL on the basis of morphological, immunological, and cytogenetic findings (60). Further, the second MIC workshop published the criteria for classification of AML (61, 62). After the fourth MIC meeting in 1989, the criteria for classification of CLL were published.

In 1999 to 2001, the WHO classification, the most recent classification for tumor of hematopoietic and lymohoid tissues, was proposed (63, 64). In an attempt to define biological homogenous entities that have clinical relevance, morphological,

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immunophenotypic, genetic and clinical features were incorporated into the classification of leukemias.

Leukemia is classified according to cell type with regard to both cell maturity and cell linage. Cell maturity is used to distinguish between acute and chronic forms of leukemia. When the malignant cells are immature (stem cells, blasts, or other immature precursors), the leukemia is classified as acute; when the cells are predominantly mature. It is described as chronic; in general these two groups correspond to rapid (acute) or slow (chronic) clinical course.

1. Acute leukemia is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of the malignant cells, which then spill over into the bloodstream and spread to other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children.

2. Chronic leukemia is distinguished by the excessive build up of relatively mature, but still abnormal, white blood cells. Typically taking months or years to progress, the cells are produced at a much higher rate than normal cells, resulting in many abnormal white blood cells in the blood. Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy. Chronic leukemia mostly occurs in older people, but can theoretically occur in any age group.

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Table 1 Classification of leukemia

Type of leukemia	Abbreviation	FAB	Alternate names
Acute myeloid	-	-	Acute nonlymphoblstic (ANLL)
Acute myeoblastic leukemia	AML	-	-
without cytologic maturation	-	M0	-
with minimal maturation	-	M1	-
with maturation	-	M2	-
Acute promyelocytic leukemia	APL	M3	Hypergranular promyelocytic
Acute myelomonocytic leukemia	AMML	M4	Naegeli-type leukemia
Acute monocytic leukemia	AMoL	M5	Schilling test leukaemia
Erythroleukemia	AEL	M6	Di Guglielmo's syndrome, erytremic myelosis
Acute megakaryoblstic leukemia	AMegL	M7	-
Acute Lymphoblastic	ALL	-	-
Precursor B-cell ALL	-	-	-
Early-Pe-B-cell-ALL	-	L1, L2	Common ALL
Pre-B-cell ALL	-	L1, L2	Common ALL
B-cell ALL	-	L3	Burkitt's leukemia
T-cell ALL	-	L2, L2	-

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Table 1 (continued)

Chronic myeloid	-	-	-
Chronic myelogenous leukemia	CML	-	Chronic granulocytic leukemia
Chronic eosinophilic leukemia	CEL	-	-
Chronic basophilic	CBL	-	-
Chronic Lymphoid	-	-	-
Chronic lymphocytic leukemia	CLL	-	-
B-cell CLL	-	-	-
T-cell CLL	-	-	-
Prolymphocytic leukemia	PLL	-	-
Hairy cell leukemia	HCL	-	Leukemic reticuloendotheliosis
Plasma cell leukemia	-	-	Multiple myeloma, leukemic phase
Sezary syndrome	-	-	Mycosis fungoides, leukemic phase

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Furthermore, leukemias are defined according to cell linage as lymphoid or myeloid. The term myeloid encompasses granulocytic, monocytic, megakaryocytic, and erythrocytic leukemias. Thus, utilizing cell maturity and cell linage, leukemia is divided into four broad categories: acute lymphoblastic leukemia (ALL), acute myeloblstic leukemia (AML: also known as acute nonlymphoblastic leukemia,

ANLL), chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML).

1.2.1.5.1 Acute myelogenous leukemia (AML)

AML begins in a single somatic hematopoietic progenitor that transforms to a cell incapable of normal differentiation. Many of these cells no longer possess the normal property of apoptosis, thus resulting in a cell with a prolonged life span and unrestricted clonal proliferation. Because the transformed cell lacks normal regulatory and growth constraints, it has a favourable competitive advantage at the expense of normal hematopoietic cells. The result is the accumulation of abnormal cells with qualitative defects. A major cause of morbidity and mortality is the deficiency of normal functioning mature hematopoietic cells rather than the presence of numerous malignant cells.

The majority of patients with acute leukemia display clinically abrupt onset of signs and symptoms of only a few weeks duration. Patients often seek medical attention because of weakness, bleeding abnormalities, or flulike symptoms. These abnormalities reflect the failure of the bone marrow to produce adequate numbers of normal cells and are caused by the proliferation and accumulation of leukemic cells in the marrow. Leukemic replacement eventually results in marrow failure and the resultant life-threatening complications of anemia, thrombocytopenia, granulocytopenia, and their sequelae. Anemia, the most consistent presenting feature, is associated with fatigue, malaise, and pallor. Hemorrhagic complications related to thrombocytopenia, and in some cases, to disseminate in intravascular coagulation (DIC) are also common. These may be mild and restricted to easy bruising, petchiae, and mucosal bleeding; or they may

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be more severe, involving gastrointestinal tract, genitourinary tract, or central nervous system (CNS) hemorrhage. Infections result from severe granulocytopenia. Bacterial infections are common (example, *Staphylococcus sp, Pseudomonas sp, Escherichia coli,* and *Klebsiella sp*) but fungal infection also occur (example, *Candida sp* and *Aspergillus sp*). Viral infections are less frequent.

In addition to the infiltration of other tissues, especially organs that play a role in fetal hematopoiesis, are often manifested by hepatosplenomegaly or lymphadenopathy, particularly in ALL and the acute monoblstic leukemia (AMoL) subtype of AML. A mediastinal mass resulting from thymic involvement is a hallmark of T-cell ALL. Gingival hypertrophy and oral lesions are primarily seen in AMoL. Bone or joint pain, caused by pressure of the expanding leukemic cell population in the marrow cavity, commonly accompanies the acute leukemias. Leukemic infiltration of the central nervous system, an ominous feature infrequently observed at initial presentation, is associated with signs and symptoms of increased intracraninal pressure (nausea, vomiting, headache, papilledema) or cranial nerve palsies. These clinical features and their relationship to pathophysiology are summarized in Table 2.

A Acute myelogenous leukemia classification

Acute myelogenous leukemia was classified to 8 major (M0 to M7) by the FAB classification which is shown in Table 3. The classification criteria are based on morphological and cytochemical features. In addition, innunophenotyping is necessary for some of the categories. The FAB classification of AML is a lineage-based morphological classification that categorizes cases according to the degree of maturation of the leukemic cells and their lineage differentiation.

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Table 2 Clinical feature of acute myelogenous leukemia (65)

Pathogenesis	Clinical manifestations
Bone marrow failure	
Anemia	Fatigue, malaise, pallor
Thrombocytopenia	Bruising, bleeding
Granulocytopenia	Fever, infections
Organ infiltration	
Marrow expansion	Bone or joint pain
Spleen	Splenomegaly
Liver	Hepatomehaly
Lymph nodes	Lymphadenopathy
Central nervous system	Neurologic symptoms
Gums, mouth	Gingival hypertrophy, oral lesions

Moreover, the MIC classification established 10 subtypes of AML which are characterized by unique cytogenetic, morphological, and immunological criteria (Table 4). These classifications address the problems of an exclusively lineage-based or an exclusively cytogenetic/molecular classification by combining the best features of both. The result is a classification that enhances clinical and diagnostic utility and retains usability.

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Table 3 FAB classification of acute myeloblastic leukemia (65)

Type	Characteristic
M0	Myeloid without cytologic maturation: Morphologically undifferentitated leukemic blasts with myeloid immunophenotype (Myeloblasts >90% of noneythroid BM cells; no Auer rods; <3% MPO+)
M1	Myeloid without maturation: Marrow leukemia cells are primarily myeloblasts with noazurophilic granules (Myeloblasts >90% of the nonerythroid BM cells; rare Auer rods; >3% MPO+)
M2	Myeloid with maturation: Leukemia cells show prominent maturation beyond myeloblast stage (Myeloblasts >30% but < 90% of the nonerythroid BM cells; frequent Auer rods)
M3	Promyelocytic: Abnormal, hypergranular promyelocytes dominate; Auer rods easily found; increased incidence of DIC (3% or more promyelocytes)
M3m	Microgranular variant of M3: Indistinct granules; nucleus often reniform or bilobed; increased incidence of DIC (3% or more promyelocytes)
M4	Myelomonocyteic: Both monocytic (monocytes and promocytes) and myeloid differentiation (maturation beyond myeloblast stage) (Immature monocytes comprising 20-80% of the nonerythroid BM cells)
M4Eo	M4with bone marrow eosinophilia: Similar to M4 with marrow eosinoplilia (abnormal and immature); associated with abnormal 16q karyotype (Eosinophilis with basophilic granules)
M5a	Monocytic, poorly differentiated: Monoblasts predominate, typically with abundant cytoplasm and single distinct nucleoli (Monoblasts comprising >80% of the nonerythroid BM cells)

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Table 3 (continued)

M5b	Monocytic, well differentiated: Predominantly promonocytes in marrow and more pronounced maturation in blood (Mixture of monoblasts and more mature monocytic cells)
M6	Erythroleukemia: Dysplastic erythroblasts with multinucleation, cytoplasmic budding, vacuolation, and megaloblastoid changes (>30% of nonerythroid cells are blasts, but >50% of marrow cells are erythroblasts)
M7	Megakaryoblastic: Wide range of morphology; cytoplasmic projections asomethimes present; electron microscopy or immunocytochemical stains necessary for diagnostic (30% or more megakaryoblasts)

Acute myeloid leukemia with minimal differentiation (M0)

Acute myeloid leukemia with minimal differentiation**,** or also known as minimally differentiated acute myeloblastic leukemia, is a subtype of AML. It is classified as M0 by FAB. It represents 2-3% of all cases of AML. Although minimally differentiated AML was recognized earlier, criteria for FAB M0 were developed in 1991 (66). The blasts in these cases cannot be recognized as myeloid based on morphology and cytochemistry, but immunophenotyping demonstrates myeloid antigens. In acute myeloblastic leukemia (M0), the blasts are agranular and nonreactive when stained for myeloperoxidase (MPO), Sudan black B (SSB) reactions, and Auer rods are not seen (58, 59, 67). In most, cases blasts are large, nuclei are round or oval, chromatin is open, nucleoli (single or multiple) are usually prominent and the cytoplasm is clear or moderately basophilic; the nucleus/cytoplasm (N/C) ratio is variable.

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Table 4 The MIC classification of AML (61)

FAB subtype	Karyotypic translocation	Frequency (%)	Suggested MIC nomenclature
AML-M2	t(8;21)(q22;q22)	12	M2/t(8;21)
AML-M3, M3m	t(15;17)(q22;q12)	10	M3/t(15;17)
AML-M5a, M5b and M 4	t/del/(11)(q23)	6	M5a/t(11q)
AML-M4Eo	inv/del(16)(q22)	5	M4Eo/inv(16)
AML-M1 and M2	t(9;22)(q34;q11)	1	M1/t(9;22)
AML-M2 or M4 with basophilia	t(6;9)(p21-22;q34)	1	M2/t(6;9)
AML-M1, M2, M4, and M7 with thrombocytosis	inv(3) (q21q26)	1	M1/inv(3)
AML-M5b with phagocytosis	t(8;16)(p11;p13)	<0.1	M5b/t(8;16)
AML-M2 with basophilia	t/del(12)(p11-13)	<0.1	M2 Baso/t(12p)
AML-M4 and M2	+4	<0.1	M4/+4

However, there are cases in which blasts may be small or pleomorphic; the N/C ratio is high, chromatin is to a large extent condensed, nucleoli are ill-defined, and the cytoplasm shows a variable degree of basophilia. The nucleus may also be lobulated or cleaved. The blasts cells react with antibodies against myeloperoxidase, CD13, CD33, and CD34. Human leukocyte antigen (HLA)-DR is positive in most patients. Occasional cases require in situ hybridization to identify the

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myeloperoxidase gene or genomic profiling for early myeloid-associated genes (68). Abnormal and unfavorable karyotypes (example, loss of the long arm of chromosome 5 (5q-) and 7 (7q-) and higher expression of the multidrug resistance glycoprotein (gp170) are frequent (69). In general, minimally differentiated acute myeloblastic leukemia has a poor prognosis (70).

Acute myeloblastic leukemia without maturation (AML-M1)

Acute myeloblastic leukemia without maturation is a quickly progressing disease in which too many immature white blood cells (not lymphocytes) are found in the blood and bone marrow. The nucleus typically has a fine, lacy chromatin pattern and distinct nucleoli. The quantity of cytoplasm is usually moderate, though this varies. In AML-M1, FAB criteria require less than 10% of the NEC to be differentiating granulocytic or monocytic cells. The peroxidase or Sudan black B reactions must demonstrate at least 3% positivity in the blast population to document myeloid differentiation. The NSE reaction is positive in less than 20% of the cells.

Acute myeloid leukemia with maturation (AML-M2)

This subtype is characterized by a translocation of a part of chromosome 8 to chromosome 21, written as t(8;21) (71). On both sides of the splice the DNA coded for different proteins, RUNX1 and ETO. These two sequences are then transcribed and translated into a single large protein, "AML-M2" which allows the cell to divide unchecked, leading to cancer.

In cases of AML-M2 subtype, the leukemic marrow infiltrate resembles M1 except evidence of maturation to or beyond the promyelocyte stage. Romanowsky-

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stained bone marrow smears show that promyelocytes and later granulocytic forms make up more than 10% of the NEC. At least 3% of the leukemic cells are peroxidase or Sudan black B positive, and usually the percentage is much higher. The non-specific esterase (NSE) activity does not exceed 20%. Moreover, they often express HLA-DR, CD13, CD15, and CD33 but the expression of CD34 and CD117 is less frequent.

The acute myeloblastic leukemias (M1 and M2 combined) are the most common types of AML. Together, M1 and M2 account for approximately 50% of cases of AML. Aside from their morphology and cytochemistry, they do not have unique features that set them apart from other myeloid subgroups. Although a translocation of chromosomes 8 and 21 (t[8;21]) has been identified in approximately 18% of patients with AML-M2 (72), this chromosome abnormality has also been found, albeit less frequently, in other FAB groups of AML. As described earlier, it is associated with the fusion gene ETO/AML1 and with a favourable prognosis, high rate of remission, and relatively long median survivals.

Acute promyelocytic leukemia (AML-M3)

Acute promyelocytic leukemia is a subtype of acute myelogenous leukemia (AML) or also known as acute progranulocytic leukemia; APL; AML with t(15;17)(q22;q12), PML-RARA, and variants. FAB subtype is M3 with its variant (M3 variant). In APL, there is an abnormal accumulation of immature granulocytes called promyelocytes. The disease is characterized by a chromosomal translocation involving the *retinoic acid receptor alpha* (*RARα* or *RARA*) gene and is unique from other forms of AML in its responsiveness to all *trans* retinoic acid (ATRA) therapy.

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Acute promyelocytic leukemia was first characterized in 1957 (73, 74). During the 1950s through 1970s APL had a 100% mortality rate and there was no effective treatment. No one knew how the cancer formed.

Morphologically, microgranular APL can be mistaken for acute myelomonocytic or monocytic leukemia. The leukemic cells appear monocytoid with prominent nuclear folding and abundant cytoplasm. The nucleus of most cells in the peripheral blood is reiform or bilobed. Granulation of these cells is scant or absent, although occasional cells with heavy granulation are almost always present. The bone marrow aspirate may reveal a morphological pattern that more closely resembles typical APL (57).

The diagnosis of microgranular of M3 (M3m) can be confirmed with cytochemical studies, including peroxidase or Sudan black B stain, which are strongly positive. The NSE reaction is usually negative, but can be positive (75). Cytogenetic studies of microgranular APL reveal the same abnormal karotype of t(15;17) that is found in the hypergranular form. Although the FAB classification defines APL solely by morphological criteria, in clinical practice the demonstration of t(15;17) by cytogenetic or molecular methods is required for confirmation of diagnosis. Only cases that are positive for the t(15;17) respond to ATRA (76).

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Acute myelomonocytic leukemia (AML-M4 or AMML)

Acute myelomonocytic leukemia (M4) is one of the most commonly diagnosed forms of AML, second only to the M2 group. The leukemic cells of M4 are characterized by both granulocytic and monocytic differentiation. On a Romanoswaky-stained smear it is usually easy to find cells with primary granules

(granulocytic differentiation) as well as cells with folded nuclei and moderate to abundant cytoplasm (monocytic differentiation). Both the peroxidase (or Sudan black B) and NSE reactions are positive in 20% to 80% of the cells. When morphological and cytochemical similarities make the distinction between M4 and M2 difficult, the diagnosis of M4 can be supported by finding a serum lysozyme level exceeding three times the normal level and a peripheral blood monocyte count of greater than 5 $x10^9$ cells/L. Some cases of M4 are associated with eosinophilia (usually 5% or more of the NEC). The eosinopils appear immature and may have large basophilic-staning granules. Unlike normal eosinopils, these cells stain positive with chloroacetate esterase 9 specific esterase) and with the PAS reaction. Moreover, the M4 variant (M4Eo) (58) is closely associated with an abnormal chromosome 16 including either a deletion (77) or inversion of the long arm (16q) (78). Patients with the 16q abnormality and bone marrow eosinophilia have a longer median survival than patients with typical M4. The molecular genetics correlation is the hybrid gene *CBFβ/MYH11*.

Acute monocytic leukemia (AML-M5)

The WHO criteria for AML-5, a patient must have greater than 20% blasts in the bone marrow, and of these, greater than 80% must be of the monocytic lineage. Furthermore, the FAB classification system divides M5 into two subtypes: poorly differentiated (M5a) and well differentiated (M5b). M5a is characterized by a predominance of monoblasts (>80%), which typically are large with abundant cytoplasm and distinct nucleoli. M5b is characterized by a spectrum of monocytic differentiation, including promonocytes (<80%) and monocytes. The peripheral

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blood usually has more monocytes than the bone marrow, where the predominant cell is the promonocyte. Monoblasts can be distinguished by having a roughly circular nucleus, delicate lacy chromatin, and abundant, often basophilic cytoplasm. These cells may also have pseudopods. By contrast, promonocytes have a more convoluted nucleus, and their cytoplasm may contain metachromatic granules. Monoblasts are typically MPO-negative and promonocytes are MPO variable. Both monoblasts and promonocytes stain positive for NSE, however NSE may often be negative. Immunophenotypically, M5-AML variably express myeloid (CD13 and CD33) and monocytic (CD11b and CD11c) markers. Cells may aberrantly express B-cell marker CD20 and the NK marker CD56. Monoblasts may be positive for CD34.

M5 is associated with characteristic chromosomal abnormalities, often involving chromosome 11 at 11q23 or t(9;11) affecting the MLL locus, however the MLL translocation is also found in other AML subtypes. MLL is believed to be prognostically unfavorable in AML-M5 compared to other genetic alterations involving MLL such as t(9;11). The t(8;16) translocation in MLL is associated with hemophagocytosis. In addition, abnormality involving the long arm of chromosome 11 (11q) have been found in about 35% of all AML-M5 and in an even higher percentage of patients with M5a. The 11q abnormality appears to be particularly associated with children with M5a (72).

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Acute erythrocytic leukemia (AML-M6)

Acute erythroid leukemia (or "acute Di Guglielmo syndrome") is a rare form of acute myeloid leukemia where the myeloproliferation is of erythroblastic precursors. Acute erythroid leukemias can be classified as follows: M6a;

erythroleukemia: both erythroid/myeloid neoplastic proliferation and M6b; pure erythroid leukemia. M6a (erythroleukemia) 50% or more of all nucleated bone marrow cells are erythroblasts, dyserythropoiesis is prominent and 30% or more of the remaining cells (non-erythroid) are myeloblasts. M6b (pure erythroid leukemia), in rare cases the erythroid lineage is the only obvious components of an acute leukemia; a myeloblast component is not apparent. The erythroid component consists of predominantly or exclusively of proerythroblasts and early basophilic erythroblasts. These cells may constitute 90% or more of the marrow elements. Despite this lack of myeloblast, these cases should be considered as acute leukemias. In WHO proposal the blastic leukeimas that are limited to the erythroid series are designated pure erythroid malignancy.

Acute megakaryoblastic leukemia (AML-M7)

Acute megakaryoblastic leukemia (AMKL) is a form of leukemia where a majority of the blasts are megakaryoblastic. This category of AML is associated with 30% or more blasts in the marrow, blast are identified as being of megakaryocyte lineage by expression of megakaryocyte specific antigens and platelet peroxidase reaction on electron microscopy. It is associated with GATA1 and risks are increased in individuals with Down syndrome (79). However, not all cases are associated with Down syndrome (80), and other genes can also be associated with AMKL (81). Another related gene is *MKL1*, which is also known as "MAL" (82). This gene is a cofactor of serum response factor (83). In adults include pancytopenia with low blast counts in the blood, myelofibrosis, an absence of lymphadenopathy and hepatosplenomegaly, poor response to chemotherapy, and short clinical course.

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In children; the same clinical presentation but with variable course especially in very young children; both leukocytosis and organomegaly may be present in children with M7. Complete remission and long term survival are more common in children than adults. In the first three years of life megakaryoblastic leukemia is the most common type of leukemia in patients with Down syndrome (84). The morphology of cells was observed by means of bone marrow smear; the immunophenotype was detected by flow cytometry and immunohistochemistry assay (85). In blood and bone marrow smears megakaryoblasts are usually medium sized to large cells with a high nuclear/cytoplasmic ratio. Nuclear chromatin is dense and homogeneous. There is scanty, variable basophilic cytoplasm which may be vacuolated. An irregular cytoplasmic border is often noted in some of the megakaryoblasts and occasionally projections resembling budding atypical platelets are present. Megakaryoblasts lack myeloperoxidase activity and stain negatively with Sudan black B. They are alpha naphthyl butyrate esterase negative and manifest variable alpha naphythyl acetate esterase activity usually in scattered clumps or granules in the cytoplasm. PAS staining also varies from negative to focal or granular positivity, to strongly positive staining. A marrow aspirate is difficult to obtain in many cases because of variable degree of myelofibrosis. More precise identification by immunophenotyping or with electron microscopy (EM), immunophenotyping using MoAb to megakaryocyte restricted antigen (CD41 and CD61) may be diagnostic (86). Prognosis depends on cause. One third of cases is associated with a t(1;22)(p13;q13) mutation in children. These cases carry a poor prognosis. Another third of cases is found in Down syndrome. These cases have a reasonably fair prognosis. The last third of cases may be heterogeneous, and carry a poor prognosis.

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1.2.1.5.2 Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is a form of leukemia, or cancer of the white blood cells characterized by excess lymphoblasts. Malignant, immature white blood cells continuously multiply and are overproduced in the bone marrow. ALL causes damage and death by crowding out normal cells in the bone marrow, and by spreading (metastasizing) to other organs. ALL is most common in childhood with a peak incidence at 2 to 5 years of age, and another peak in old age. The overall cure rate in children is about 80%, and about 45% to 60% of adults have long-term disease-free survival. Initial symptoms are not specific to ALL, but worsen to the point that medical help is sought. The signs and symptoms of ALL are variable but follow from bone marrow replacement and/or organ infiltration. The signs and symptoms of ALL result from the lack of normal and healthy blood cells because they are crowded out by malignant and immature leukocytes (white blood cells). Moreover, people with ALL experience symptoms from malfunctioning of their erythrocytes (red blood cells), leukocytes, and platelets. Laboratory tests which might show abnormalities include blood count tests, renal function tests, electrolyte tests, and liver enzyme tests.

Diagnosing ALL begins with a medical history and physical examination, complete blood count, and blood smears. Because the symptoms are so general, many other diseases with similar symptoms must be excluded. Typically, the higher the white blood cell count, the worse the prognosis. Blast cells are seen on blood smear in 90% of cases. A bone marrow biopsy is conclusive proof of ALL. A lumbar puncture (also known as a spinal tap) will tell if the spinal column and brain has been invaded.

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Pathological examination, cytogenetics (particularly the presence of Philadelphia chromosome) and immunophenotyping, establish whether the "blast" cells began from the B lymphocytes or T lymphocytes. DNA testing can establish how aggressive the disease is; different mutations have been associated with shorter or longer survival. Beside, Immunohistochemical testing may reveal Terminal deoxynucleotidyl transferase (TdT) or CALLA antigens on the surface of leukemic cells. TdT is a protein expressed early in the development of pre-T and pre-B cells while CALLA is an antigen found in 80% of ALL cases and also in the "blast crisis" of CML. Medical imaging (such as ultrasound or CT scanning) can find invasion of other organs commonly the lung, liver, spleen, lymph nodes, brain, kidneys, and reproductive organs. Subtyping of the various forms of ALL used to be done according to the FAB (Table 5), which was used for all acute leukemias. Each subtype is then further classified by determining the surface markers of the abnormal lymphocytes, called immunophenotyping. There are 2 main immunologic types: pre-B cell and pre-T cell. The mature B-cell ALL (L3) is now classified as Burkitt's lymphoma/leukemia. Subtyping helps determine the prognosis and most appropriate treatment in treating ALL.

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1.2.1.5.3 Chronic myelogenous leukemia (CML)

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Chronic myelogenous (or myeloid) leukemia (CML), also known as chronic granulocytic leukemia (CGL), is a form of leukemia characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood.

Table 5 FAB classification of acute lymphoblastic leukemia (65)

Cytologic features	Acute lymphoblastic leukemia		
	L1	**L2**	**L3**
Cell size	Predomianatly small	Large , heterogeneous	Large, homogeneous
Nuclear chromatin	Homogeneous in any one case	Heterogeneous	Finely stippled, and homogeneous
Nuclear shape	Regular, occasional clefting	Irregular, clefting and indentation common	Regular (round to oval)
Nucleoli	Inconspicuous	One or more, often large	One or more, prominent
Cytoplasm	Scanty	Variable; often moderately abundant	Moderately abundant; strongly basophilic
Cytoplasmic vacuolation	Variable	Variable	Prominent

CML is a clonal bone marrow stem cell disorder in which proliferation of mature granulocytes (neutrophils, eosinophils, and basophils) and their precursors is the main finding. It is a type of myeloproliferative disease associated with a characteristic chromosomal translocation called the Philadelphia chromosome. It is now treated with imatinib and other targeted therapies, which have dramatically improved survival. Patients are often asymptomatic at diagnosis, presenting incidentally with an elevated white blood cell count on a routine laboratory test. In this setting, CML must be

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distinguished from a leukemoid reaction, which can have a similar appearance on a blood smear.

Symptoms of CML may include enlarged spleen, malaise, low-grade fever, gout, increased susceptibility to infections, anemia, and thrombocytopenia with easy bruising (although an increased platelet count (thrombocytosis) may also occur in CML) (87, 88).

CML is often suspected on the basis on the complete blood count (CBC), which shows increased granulocytes of all types, typically including mature myeloid cells. Basophils and eosinophils are almost universally increased; this feature may help differentiate CML from a leukemoid reaction.

Bone marrow biopsy is often performed as part of the evaluation for CML, but bone marrow morphology alone is insufficient to diagnose CML (87-89). Ultimately, CML is diagnosed by detecting the Philadelphia chromosome. This characteristic chromosomal abnormality can be detected by routine cytogenetics, by fluorescent *in situ* hybridization, or by PCR for the *bcr-abl* fusion gene (88). Controversy exists over so-called Ph-negative CML, or cases of suspected CML in which the Philadelphia chromosome cannot be detected. Many such patients in fact have complex chromosomal abnormalities which mask the (9;22) translocation, or have evidence of the translocation by FISH or RT-PCR in spite of normal routine karyotyping (90). The small subset of patients without detectable molecular evidence of bcr-abl fusion may be better classified as having an undifferentiated myelodysplastic/myeloproliferative disorder, as their clinical course tends to be different from patients with CML (91).

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1.2.1.5.4 B-cell chronic lymphocytic leukemia (B-CLL)

B-cell chronic lymphocytic leukemia (B-CLL), also known as chronic lymphoid leukemia (CLL), is the most common type of leukemia. CLL affects B cell lymphocytes. B cells originate in the bone marrow, develop in the lymph nodes, and normally fight infection by producing antibodies. In CLL, the DNA of a B cell is damaged, so that it can't produce antibodies. Additionally, B cells grow out of control and accumulate in the bone marrow and blood, where they crowd out healthy blood cells. CLL is a stage of small lymphocytic lymphoma (SLL), a type of B-cell lymphoma, which presents primarily in the lymph nodes (64). CLL and SLL are considered the same underlying disease, just with different appearances. CLL is a disease of adults, but, in rare cases, it can occur in teenagers and occasionally in children (inherited). Most (>75%) people newly diagnosed with CLL are over the age of 50, and the majority are men. Most people are diagnosed without symptoms as the result of a routine blood test that returns a high white blood cell count, but, as it advances, CLL results in swollen lymph nodes, spleen, and liver, and eventually anemia and infections. Early CLL is not treated, and late CLL is treated with chemotherapy and monoclonal antibodies. DNA analysis has distinguished two major types of CLL, with different survival times. CLL that is positive for the marker ZAP-70 has an average survival of 5 years. CLL that is negative for ZAP-70 has an average survival of more than 25 years. Patients with slowly-progressing disease can be reassured and may not need any treatment in their lifetimes (92). Recent publications suggest that two (93) or three (94) prognostic groups of CLL exist based on the maturational state of the cell. This distinction is based on the maturity of the lymphocytes as discerned by the immunoglobulin variable-region heavy chain (IgV_H)

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gene mutation status (95). High risk patients have an immature cell pattern with few mutations in the DNA in the IgV_H antibody gene region whereas low risk patients show considerable mutations of the DNA in the antibody gene region indicating mature lymphocytes. Since assessment of the IgV_H antibody DNA changes is difficult to perform, the presence of either CD38 or Z-chain–associated protein kinase-70 (ZAP-70) may be surrogate markers of high risk subtype of CLL (95). Their expression correlates with a more immature cellular state and a more rapid disease course.

In addition to the maturational state, the prognosis of patients with CLL is dependent on the genetic changes within the neoplastic cell population. These genetic changes can be identified by fluorescent probes to chromosomal parts using a technique referred to as fluorescent *in situ* hybridization (FISH) (95). Four main genetic aberrations are recognized in CLL cells that have a major impact on disease behaviour (96).

1. Deletions of part of the short arm of chromosome 17 (del 17p), which target the cell cycle regulating protein p53 are particularly deleterious. Patients with this abnormality have significantly short interval before they require therapy and a shorter survival. This abnormality is found in 5–10% of patients with CLL.

2. Deletions of the long arm on chromosome 11 (del 11q) are also unfavorable although not to the degree seen with del 17p. The abnormality targets the Ataxia telangiectasia mutated (ATM) gene and occurs infrequently in CLL (5–10%).

3. Trisomy 12, an additional chromosome 12, is a relatively frequent finding occurring in 20–25% of patients and imparts an intermediate prognosis.

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4. Deletion of the long arm of chromosome 13 (del 13q) is the most common abnormality in CLL with roughly 50% of patients with cells containing this defect. These patients have the best prognosis and most will live many years, even decades, without the need for therapy. The gene targeted by this deletion is a segment coding for microRNAs miR-15a and miR-16-1.

In the past, cases with similar microscopic appearance in the blood but with a T cell phenotype were referred to as T-cell CLL. However, it is now recognized that these so-called T-cell CLLs are in fact a separate disease group and are currently classified as T-cell prolymphocytic leukemias (97). CLL should not be confused with acute lymphoblastic leukemia, (ALL) a highly aggressive and highly treatable leukemia most commonly diagnosed in children.

Most people are diagnosed without symptoms as the result of a routine blood test that returns a high white blood cell count. Less commonly, CLL may present with enlarged lymph nodes without a high white blood cell count or no evidence of the disease in the blood. This is referred to as small lymphocytic lymphoma. In some individuals the disease comes to light only after the neoplastic cells overwhelm the bone marrow resulting in anemia producing tiredness or weakness.

The disease is easily diagnosed. CLL is usually first suspected by the presence of a lymphocytosis, an increase in one type of the white blood cell, on a complete blood count (CBC) test. This frequently is an incidental finding on a routine physician visit. Most often the lymphocyte count is greater than 4,000 cells/µL of blood but can be much higher. The presence of a lymphocytosis in an elderly individual should raise strong suspicion for CLL and a confirmatory diagnostic test, in particular flow cytometry, should be performed unless clinically unnecessary. The

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diagnosis of CLL is based on the demonstration of an abnormal population of B lymphocytes in the blood, bone marrow, or tissues that display an unusual but characteristic pattern of molecules on the cell surface. This atypical molecular pattern includes the co-expression of cells surface markers CD5 and CD23. In addition, all the CLL cells within one individual are clonal, that is genetically identical. In practice, this is inferred by the detection of only one of the mutually exclusive antibody light chains, kappa or lambda, on the entire population of the abnormal B cells. Normal B lymphocytes consist of a stew of different antibody producing cells resulting in a mixture of both kappa and lambda expressing cells. The lack of the normal distribution of kappa and lambda producing B cells is one basis for demonstrating clonality, the key element for establishing a diagnosis of any B cell malignancy (B cell Non-Hodgkin lymphoma). The combination of the microscopic examination of the peripheral blood and analysis of the lymphocytes by flow cytometry to confirm clonality and marker molecule expression is needed to establish the diagnosis of CLL. Both are easily accomplished on a small amount of blood. A flow cytometer is an instrument that can examine the expression of molecules on individual cells in fluids. This requires the use of specific antibodies to marker molecules with fluorescent tags recognized by the instrument. In CLL, the lymphocytes are genetically clonal, of the B cell lineage (express CD19 and CD20), and characteristically express the marker molecules CD5 and CD23. These B cells resemble normal lymphocytes under the microscope, although slightly smaller, and are fragile when smeared onto a glass slide giving rise to many broken cells (smudge cells).

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Hematologic disorders that may resemble CLL in their clinical presentation, behavior, and microscopic appearance include mantle cell lymphoma, marginal zone lymphoma, B cell prolymphocytic leukemia, and lymphoplasmacytic lymphoma.

1. B cell prolymphocytic leukemia (B PLL), is a related but more aggressive disorder, has cells with similar phenotype but that are significantly larger than normal lymphocytes and have a prominent nucleolus. The distinction is important as the prognosis and therapy differs from CLL.

2. Hairy cell leukemia is also a neoplasm of B lymphocytes but the neoplastic cells have a distinct morphology under the microscope (hairy cell leukemia cells have delicate, hair-like projections on their surface) and unique marker molecule expression. All the B cell malignancies of the blood and bone marrow can be differentiated from one another by the combination of cellular microscopic morphology, marker molecule expression, and specific tumor-associated gene defects. This is best accomplished by evaluation of the patient's blood, bone marrow and occasionally lymph node cells by a pathologist with specific training in blood disorders. A flow cytometer is necessary for cell marker analysis and the detection of genetic problems in the cells may require visualizing the DNA changes with fluorescent probes by FISH.

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1.2.2 *Wilms' tumor 1* gene, *WT1*

The Wilms tumor also known as neoblastoma, is a cancer of kidney firstly described in 1899 by Max Wilms in Wilms tumor patients with WAGR (Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation) syndrome and deletion of chromosome 11q13 (16, 17, 98). Wilms' tumor was recognized as a tumor suppressor more than 15 years ago. This tumor is also observed in an association with several rare

malformation syndromes: (a) WAGR syndrome linked to a constitutional 11p13 deletion (16, 99); (b) Denys-Drash syndrome (diffuse mesangial sclerosis, male pseudohermaphroditism, and WT); and (c) Beckwith-Wiedemann syndrome (gigantism, macroglossia, omphalocele, hyperinsulinism, and predisposition to several tumors such as WT, adrenocortical carcinoma and rhabdomyosarcoma).

1.2.2.1 Structure and function of WT1

The human *WT1* gene is located at chromosome 11p13 which contains 10 exons. The *WT1* gene encodes 48-57 kDa (98) WT1 protein which comprises two domains. The N-terminal is proline and glutamine-rich as well as having transcriptional regulatory function, and is involved in RNA and protein interaction. The C-terminal DNA-binding domain, exon 7-10 code consists of four Cys_2His_2 zinc fingers, which permit binding to target DNA sequences but are also involved in RNA and protein interactions (Figure 1).

Additionally, the zinc fingers bind to DNA target sequences that have the consensus sequence 49-GCGTGGGAGT-39. The gene spans 50 kb of genomic DNA containing 10 exons that code for a 3.2 kb mRNA. Four transcripts are expressed from the WT1 locus that are generated by alternative splicing affecting a single entire exon, exon 5 (Splice1) and the 39 end of exon 9 (Splice 2) (100). Splicing in exon 5 removes an exon encoding 17 amino acids and splicing in exon 9 generates an insertion of three amino acids-lysine, threonine, and serine (KTS) that alters the DNA-binding specificity of the zinc-finger domain. The four predominant WT1 isoforms generated *in vivo* are termed +/+ (both splices 1 and 2 are present) +/- (splice 1 present only), -/+ (splice 2 present only), and -/- (neither splice present).

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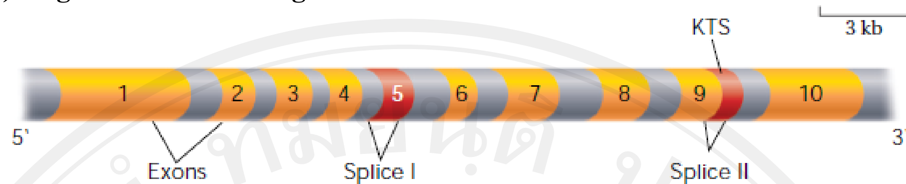
(a) Organization of *WT1* gene



(b) Structure of WT1 protein

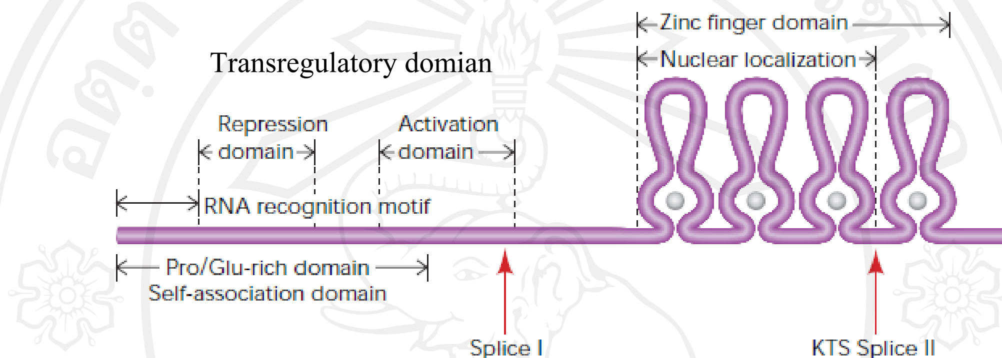


Figure 1 Structure of (a) *WT1* gene and (b) WT1 protein (101)

These four isoforms have molecular masses in the range of 52 to 54 kDa. Additional functional WT1 protein isoforms are generated by RNA editing at codon 280 within exon 6 and by alternate translation initiation codon usage at two positions (102-104). Translation initiation at a CUG upstream of the initiation AUG generates four larger WT1 proteins of 60-62 kDa. Translation initiation at a second AUG generates amino-terminally truncated WT1 proteins of molecular masses of 36-38 kDa. These additional WT1 protein isoforms have not yet been extensively investigated, although they do have the capacity to regulate transcription (102-105) as do the four major protein isoforms. The structure of the WT1 protein, including its functional motifs, and possible protein isoforms is shown in Figure 2. Two functional domains for transcriptional repression (exon 1) and transcriptional activation (exons 3 and 4)

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have also been identified (106). WT1 protein is also able to form a transitory interaction through the carboxy-terminal zinc fingers with the splicing factor U2AF65 and with RNA (25, 107). Additional interactions with RNA are mediated via an RNA recognition motif (RRM) near the WT1 amino terminus that is similar to an RRM in the constitutive splicing factor, UIA (108). WT1 was the first tumor suppressor gene identified in Wilms' tumor, a pediatric malignancy affecting the kidney (109-111). Allele loss and mutation analysis have shown that 10% of sporadic Wilms' tumors carry homozygous inactivating mutations affecting WT1 in accordance with the Knudson two-hit hypothesis for tumor suppressor gene inactivation. The Wilms' tumor mutations are commonly truncating mutations that delete part or all of the zinc-finger region (112-114). These are postulated to disrupt binding of the WT1 zinc fingers to sites within the promoters of critical growth-regulating genes. Numerous *in vitro* studies have demonstrated that WT1 is a transcription factor. WT1 isoforms with Splice 1 and 2 differences have distinct DNA-binding and transcriptional activities (115-119). WT1 target genes include genes involved in cellular proliferation, differentiation, apoptosis, and sex determination. These are members of the IGF family, EGFR, TGFB, BCL2, PDGFA, CSF1, Amphiregulin, E-cadherin, ornithine decarboxylase, p21, hTERT, and DAX-1 (24–36). WT1 + KTS and - KTS isoforms can also form direct functional interactions with components of the nuclear spliceosome machinery, suggesting that they also play a role in post-transcriptional gene regulation. + KTS isoforms preferentially co-localize in the nucleus with splicing factors whereas - KTS isoforms associate with DNA in transcription factor domains; however, these associations are not mutually exclusive (25, 26, 120). + KTS isoforms can also bind to RNA (107, 108).

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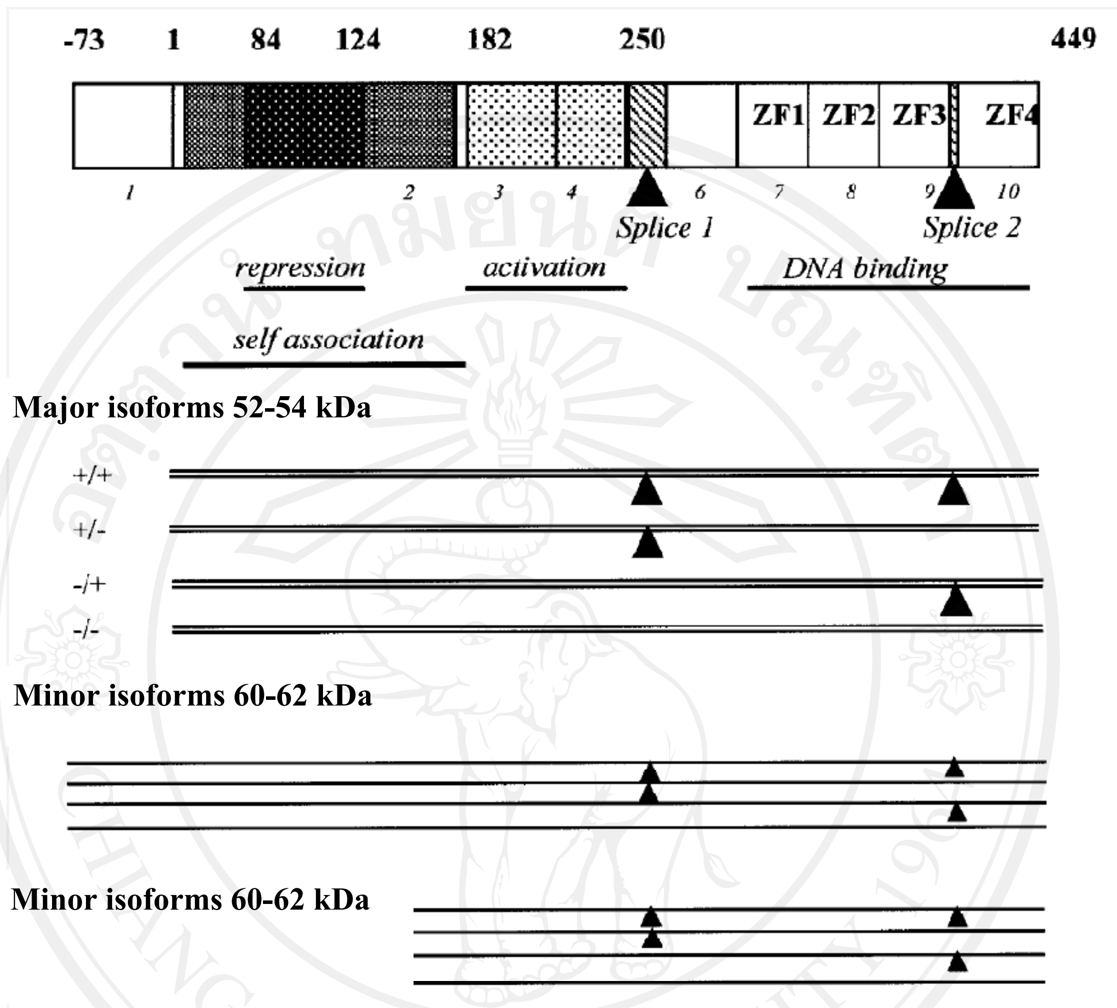

Figure 2 Diagrammatic representation of major and minor WT1 protein isoforms. Numbers in boldface represent codon numbers defining functional domains of the protein. Shaded boxes denote self-association, repression, and activation domains. splice site 1 encoding 17 amino acids and splice site 2 encoding 3 amino acids are depicted by solid triangles and striped boxes. Exon numbering is denoted by italicized numbers. The major 52-54 kDa WT1 isoforms start at codon 1, the minor isoforms of 60-62 kDa at codon 273 and the short 36-38 kDa isoforms at codon 127. Additional minor isoforms containing LEU to PRO substitution in exon 6, that are generated by RNA editing, are found in human testes (121).

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1.2.2.2 Normal expression of WT1

WT1 gene is an important regulatory molecule involved in cell growth and development. It is one of at least three genes that are involved in the development of Wilms tumor, a paediatric kidney cancer. The expression pattern of the gene indicates that WT1 does not only play a role during kidney development but is also involved in the development and homeostasis of several other tissues. The physiological function of this gene, however, remains to be elucidated. The gene products have been implicated in many processes such as proliferation, differentiation, and programmed cell death (apoptosis). In embryo development, WT1 expression is primarily found in the urogenital system (122, 123). It is required for kidney development and is expressed at the onset of nephrogenesis in the uninduced metanephric mesenchymal cells; expression levels are strongest during condensation of the mesenchyme. In adult tissue, WT1 expression is also found in the urogenital system, the central nervous systems, and in tissues involved in hematopoiesis which include bone marrow and lymph nodes.

The WT1 proteins function as transcription factors but may additionally be involved in splicing. Disruption of these activities may lead to aberrant development. In this paper we will discuss the role of the *WT1* gene during normal development and homeostasis of several tissues.

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1.2.2.3 WT1 in normal hematopoiesis

In normal bone marrow, WT1 is expressed at low levels in $CD34^+$ hematopoietic progenitor cells as well as downregulation of differentiation. Gaiger *et al.* (2000) demonstrated that 7/9 AML cells had higher levels of WT1 expression than normal

$CD34^+$ cells (124). WT1 expression has also been examined in individual marrow $CD34^+$ cells (35). Using two different single cell RT-PCR techniques, 1.2% of $CD34^+$ cells expressed WT1. The level of WT1 expression in single $CD34^+$ cells was comparable to levels in K562 cells. Moreover, both $CD34^+/CD38^-$ and $CD34^+/CD38^+$ had similar frequencies of WT1 expression. WT1 can be detected in $CD34^+$ cells isolated from cord blood and fetal blood mononuclear cells (37, 125). WT1 levels were five-fold higher in more primitive progenitor cells ($CD34^+$, rho dull) than in more differentiated cells ($CD34^+$, rho bright). *In vitro* progenitor assays may help clarify WT1 expression in $CD34^+$ cells. Most (28/35) individual colonies derived from human fetal blood expressed WT1 on day 14, but WT1 was only rarely detected on day 21 (1/20) (37, 125). The 12-day cultures of $CD34^+$/WT1 in normal marrow cells with granulocyte colony stimulating factor plus stem cell factor resulted in granulocytic maturation with rapid loss of WT1 expression (36). Consequences of forced WT1 expression in progenitor cells have been reported. Compared to normal murine marrow cells, induced constitutive expression of human WT1 results in enhanced proliferation (colony formation) but decreased differentiation (as detected by decreased maturation-related surface markers) (126). Expression of WT1 in cord blood $CD34^+$ cells forced by retroviral gene transfer reduced proliferation (127, 128). Differentiation was enhanced in one study and not impaired in another. In contrast, expression of WT1 in cord blood $CD34^+$, $CD38^-$ cells results in cellular quiescence (127). Divergent effects of WT1 expression on early and late progenitors suggest that WT1 may have a hemopoietic regulatory function. The PCR and colony-forming studies indicate that there is a low frequency of normal marrow $CD34^+$ cells that have WT1 levels comparable to leukemic cells. The lack of WT1 expression in $CD34^+$

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negative cells is interesting, since there appears to be $CD34^+$ negative human stem cells (129). Moreover, the expression of WT1 in hematopoietic progenitor cells by placing WT1 is downstream of the *tec* promoter. This resulted in increased bone marrow cellularity and increased number of BFU-E, CFU-GM, and CFU-GEMM. However, the production of mature cells was normal in mice. It is of note that these mice did not develop spontaneous leukemia. However, when the bone marrow was infected with virus carrying AML-ETO and then developed to leukemia.

Finally, the role of WT1 in human hematopoistic cells, WT1 appears to behave as a tumor suppressor gene as the overexpression in early human bone marrow cells. During hematopoiesis in normal blast cells, WT1 was presented in early hematopoiesis blast cells but downregulated in cell expressing $CD33^+$. Moreover, WT1 plays a role in differentiation and proliferation of hematopoietic cells.

1.2.2.4 WT1 in leukemia

The original clone of WT1, WT33 was isolated from a B cell leukemia cell line (16). Subsequently, WT1 was observed to be widely expressed in both myeloid and lymphoid leukemic cell lines. They are also expressed 80% in lymphoid luekemias, acute lymphocytic leukemia (ALL) and 90% in myeloid leukemia, acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML) in blast crisis and myelodysplastic syndrome (MDS) when compared to normal bone marrow and peripheral blood. The highest levels of WT1 expression occur in leukemia with immature phenotype including chronic myelogenous leukemia in blast crisis, while normal peripheral blood mononuclear cells express virtually undertectable levels of WT1. The expression of WT1 in leukemia is independent of age, karyotype, or FAB

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subtype, with the exception that M5 acute myelocytic leukemia is less likely to express WT1 (32).

WT1 is expressed at high levels in the majority of cases of acute leukemia (5, 38, 39). Whether this finding reflects a potential oncogenic role for WT1 in this disorder or simply reflects the transformation of cells that ordinarily express this gene is a subject of some controversy. Point mutations in WT1 have been identified in approximately 10% of patient with myeloid leukemia (125, 130). This finding further complicates the interpretation of the role of WT1 in leukemia. WT1 is also used as a prognostic factor and marker for MDS from refractory anemia (RA) with excess of blast (RAEB) in transformation (RAEB-t) *via* RAEB in cases of acute leukemia (21, 23). Increasing of WT1 expression in peripheral blood clearly indicated that leukemic change of MDS was impending. Recently it was reported that in paroxysmal nocturnal hemoglobinuria, some case of which progressed to leukemia, expression of WT1 in bone marrow was at a high level and that patients with the highest levels of WT1 expression progressed to be overt leukemia within a short period (131). Early reports used semiquantitative RT-PCR to measure WT1 mRNA expression, normalized to the K562 cell line. Inoue *et al.* (1994) demonstrated increased expression relative to normal bone marrow in AML and ALL and higher expression in CML blast crisis than in chronic phases but no difference in lymphoma cases compared to bone marrow (5). Moreover, King-Underwood and Pritchard-Jones studied a total of 67 cases of acute leukemias by using single-strand conformation polymorphism (SSCP) analysis and identified mutations in many them which included 14% of their AML patients and 20% of their patients with biphenotypic leukemia (125, 130).

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1.2.3 Curcumin

1.2.3.1 Curcumin structure

Curcuminoids is a curcumin or a derivation of curcumin with different chemical groups. It is a yellow-colored polyphenol derived from the rhizome of the turmeric. Its scientific name is *Curcuma longa* Linn which is a member of Zingiberaceae or ginger family. It was given-many names such as turmeric in English, haldi in Hindi and ukon in Japanese. Also, it has many common names in Thailand, such as Khamin (general), Khamin Kaeng, Khamin yok, Khamin hua (Chiang Mai), Khamin chan (central peninsula), Taa-yo (Karen-Kamphaenpet), and Sa-yo (Karen-mae Hong Son) (132). The turmeric has much branching, bright orange within. Its leaves emerge directly from the underground rhizome with overlapping petioles 8-15 cm long or more. Its flowers are pale yellow, expect in the upper part whereas the bracts are white and green or pink (Figure 3). Curcumin was first isolated in 1819. Its active ingredient was first isolated in 1842 by Vogel. In 1870, it was crystallized and identified as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1*E*,6*E*) (133). In 1810, curcumin structure was delineated as diferuloylmethane containing low molecular weight polyphenol. Then it was synthesized and confirmed in 1913 (134). Curcumin has a molecular weight (MW) of 368.37 and a melting point of 183°C. On ultraviolet-visble spectrophotometic investigation, maximum light absorption of curcumin occurs at 420 nm. In addition, commercial grade curcumin contains approximately 77% diferuloylmethane (curcumin I, MW 368), 17% demethoxycurcumin (curcumin II, MW 338), and 6% bisdemethoxycurcumin (curcumin III, MW 308). All these analoges suggest that hydroxyl groups in curcumin are required for its antioxidant activity, while methoxy groups are essential for its anti-inflammatory and antiproliferative activity.

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Figure 3 A stemless rhizomatous of *Curcuma longa*. Leaves emerge directly from the underground with overlapping petioles 8-15 cm long or more. They have ellipse-shaped or elongated lance-shaped blades. The plant consists of large pale green, pouch like, curved bract, each with two or more pale yellow or pale pink flowers. The rhizome is bright orange or yellow within and scented (135).

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1.2.3.2 Solubility and stability

In addition, curcuma species contain turmerin (a water-soluble peptide), essential oils (such as turmerones, atlantones, and zingiberene) and curcuminoids including curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. Curcuminoids can be defined as phenolic compounds derived from the root of *Curcuma* spp. (Zingiberaceae). Curcumin has a wide spectrum of biological and

pharmacological activities. Chemically, curcumin is a bis-α,β-unsaturated β-diketone (commonly called diferuloylmethane, Figure 4), which exhibits keto-enol tautomerism having a predominant keto form in acidic and neutral solutions and stable enol form in alkaline medium and in the cell membrane (136). However, curcumin acts as an extraordinarily potent H-atom donor at pH 3-7. This is due to in the keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings contains a highly activated carbon atom, and the C-H carbon bounds on this carbon are very weak because the unpaired electron delocalises on the adjacent oxygens (Figure 5). In contrast, above pH 8, the enolate form of the heptadienone chain predominates, and curcumin acts mainly as an electron donor which a mechanism more typical for the avenging activity of phenolic antioxidant (137). Thus, curcumin is relatively insoluble in water but dissolves in acetone, dimethylsulphoxide (DMSO), chloroform and ethanol (138). Moreover, curcumin is unstable at basic pH and degrades within 30 min to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hxanal, ferulic acid, feruloylmethane and vanillin (139). The presence of fetal calf serum or human blood, or addition of antioxidants such as ascorbic acid, N-acetylcystine or glutathione, completely blocks this degradation in culture medium or phosphate buffer above pH 7 (136). Furthermore, under acidic conditions the curcumin can be degraded slowly, with less than 20% of total curcumin decomposed at 1 h (136). Moreover, the curcumin is also found that it is more stable in cell culture medium containing 10% fetal calf serum or in human blood but 50% of curcumin had still decomposed after 8 h and less that 20% decomposition within 1 h as compared to 90% within 30 min in serum-free medium (136).

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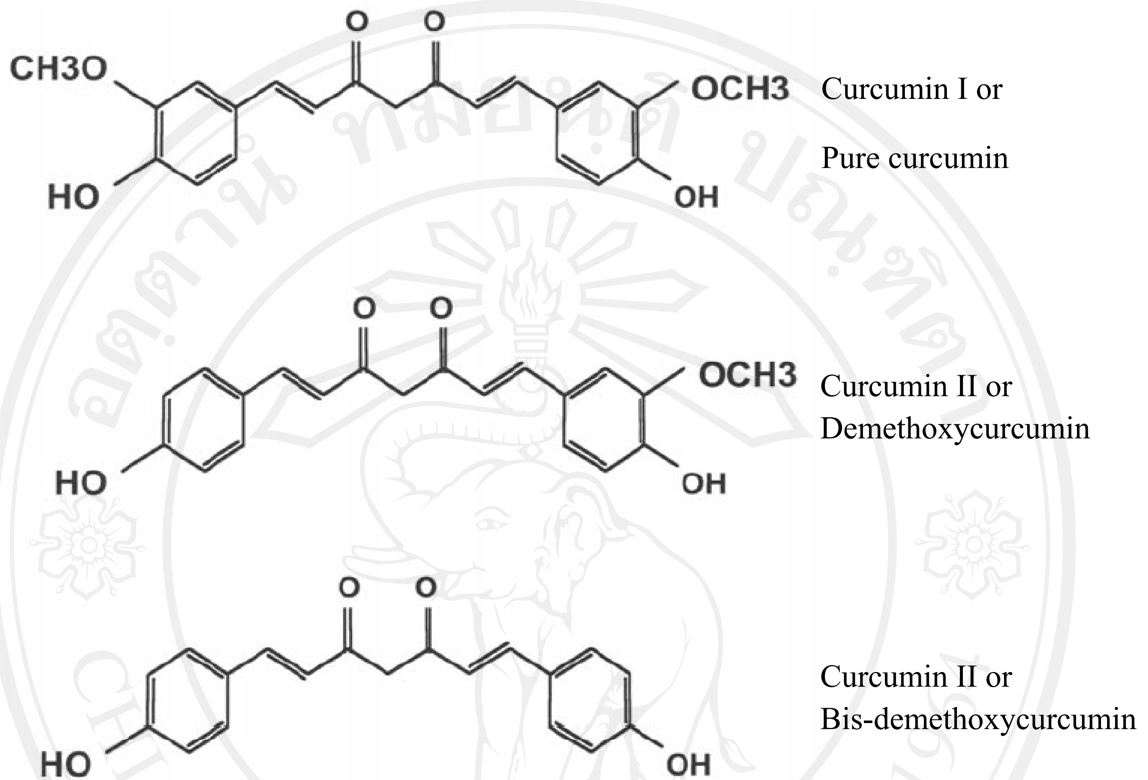

Figure 4 Nature yellow dye, Curcumin (diferuloylmethane; 1, 7-Bis(4-hydrooxy-3-methoxypheneyl)-1,6-heptadine-3,5-dione) curcumin I, MW 368; curcumin II, MW 338; Curccumin III, MW 308 (140) and keto-enol tautomers of curcumin (141).

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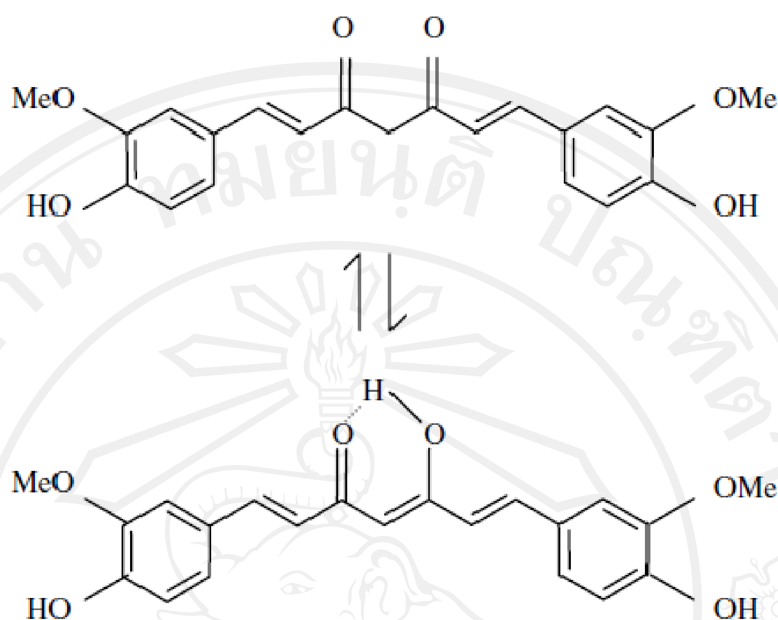

Figure 5 Tautomerism of curcumin under physiological conditions. Under acidic and neutral conditions, the bis-keto form (top) predominates, whereas the enolate form is found above pH 8.0 (142).

1.2.3.3 Metal-binding chemistry

Metal ion complexation of curcumin, which consists of the cations $[VO]^{2+}$ (143, 144), Mn^{2+} (145, 146), $Fe^{2+/3+}$ (147, 148), and Cu^{2+} (149-151), has a particular biological interest. Unfortunately, the metal ion complexation reactions of curcumin and characterization of complexes are most often conducted in nonionizing solvents such as alcohols. Studies of aqueous metal ion speciation with curcumin under controlled pH conditions are of greater relevance to the biological environment. Because the acetylacetonate (acac-) ligand (region A of curcumin, Figure. 6) is known to bind all the above metal ions, and the acac complexes have some kinetic or

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thermodynamic stability in polar and/or protic media (141), it is reasonable to assume that the interactions of these ions with curcumin *in vivo* is biologically significant. Iron is the metal ion whose aqueous speciation with curcumin has been studied most thoroughly (148). Interestingly, although the speciation studies were conducted at pHs that ranged to well over 7, there was rarely mention of the decomposition of curcumin at pH >7, nor were the appropriate checks for its decomposition made. The principal conclusion of the iron speciation work is that species such as [FeIII (H_2curcumin-)$(OH)_2$] are readily found at pH 7 (148). These have formation constants nearly as great as that of transferrin (log Kf = 22.06). This formation constant must be compared to the pKa of curcumin (here indicated as H_3curcumin), which is 8.54. The values for Fe(II) are around 12 orders of magnitude smaller than those of Fe(III). There is no doubt that curcumin is a strong chelator of iron under neutral to slightly acidic conditions, although less is known of its affinity to other biometals. The unique molecular structures of the curcumin have been showed to be endowed with beneficial biological activities including antioxidant action, anti-inflammatory action, anticarcinogenic action and anti-mutagen activity (Figure 6). The complex kinetics degradation of curcumin is dependent pH in aqueous solution. Indeed, another study found pseudo-zero-order kinetics of curcumin decomposition when working in unbuffered aqueous medium of pH 10 –13.5, with a rate constant of 1.39 x 10^{-9} M/min (152). A recent study showed first order kinetics of degradation for curcumins I, II and III, where phosphate, borate, Tris and carbonate buffers were employed (153). Curcumin III (bisdemethoxycurcumin) was the most stable, with a rate order of I > II > III. Curcumin is also photodegradable, as studied in isopropanol solution (154). A number of studies of pharmacology and metabolites of curcumin exist (155-157). The intestinal metabolites in human and rats

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have been identified as curcumin glucuronide, curcumin sulfate, tetrahydrocurcumin and hexahydrocurcumin (157). Thus, both metabolic conjugation and reduction are observed. Traces of the aforementioned decomposition products of dihydroferulic acid and ferulic acid were found as biliary metabolites of oral curcumin administration in rats (158), which indicates that the chemical decomposition products of curcumin are present *in vivo*, and may be relevant to biological activity.

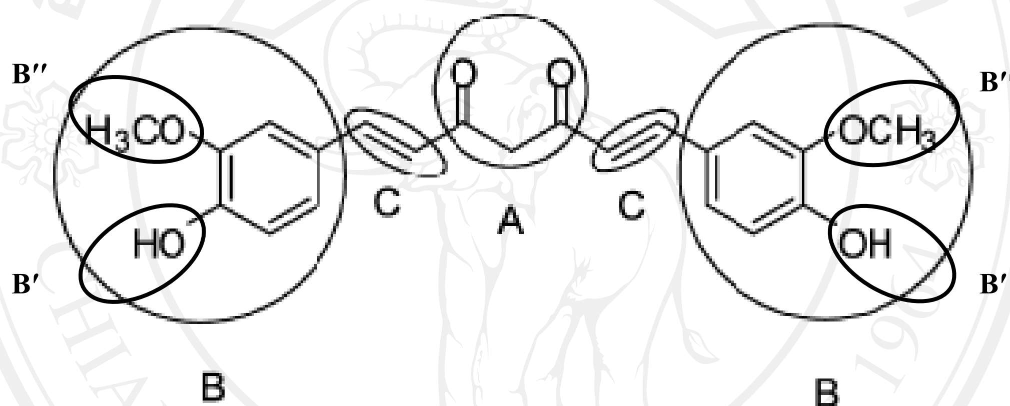

Figure 6 Nomenclature of regions of curcumin I. (A) β-diketone or keto-enol, (B) Phenolic, (B′) Parahydroxy group and (B″) methoxy group in phenolic region, (C) alkene linker (141). Curcumin has been shown to be endowed with several biological activities potentially useful for clinical use. These biological activities are attributed their unique molecular structure:

A: Ketone groups; anti-inflammatiory, anticancer and anti-mutagen activity

B′: Parahydroxy groups; antioxidant activity

B″: Methoxy group; anticancer

C: Double bound; anti-inflammatory, anticancer and anti-mutagen activity

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1.2.3.4 History and traditional uses of curcumin

Curcumin has been used in Asian medicine since the second millennium. The applications are referred to in the ancient Hindu scripture, the Ayurveda Unaia, Siddha, and Thailand. Besides, their aromatic, stimulant, and colouring properties in the diet, turmeric can be mixed with other natural compounds such as slaked lime, and has been used topically as a treatment for wounds, inflammation and tumours. In contrast to the maximum dietary consumption of 1.5 g per person per day in certain South East Asian communities, smaller quantities of turmeric tend to be used for medicinal purposes (159). The appeal of turmeric in food preservation colouring and flavouring is global according to the Food and Agriculture Organization of United Nations; over 2400 metric tons of turmeric are imported annually in to the USA for consumer use. Furthermore, turmeric has been used for many ailments, particularly as an anti-inflammatory agent, and curcumin has been identified as the active principle of turmeric (140). Furthermore, curcumin has been shown that antioxidant, anti-infammatory (142, 160-162), antimicrobial, and anticarcinogenic activities (163-167). In addition, the hepato-protective and nephro-protective (168-170), thrombosis suppressing (171), myocardial infarction protective (172), hypoglycaemic (173-175) and antirheumatic (176) effect of curcumin are also well established. Many studies have shown that curcumin is extremely safe even at very high dose in both animal and human models (177-181).

Moreover, the study of three different phase I clinical trials indicated that curcumin, when taken as high as 12 g per day, is well tolerated (177, 178, 181). Similarly, the efficacy of curcumin in various diseases including cancer has been well established (182). Several clinical studies dealing with the efficacy of curcumin in

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humans can also be cited (182, 183). The pharmacological safety and efficacy of curcumin makes it a potential compound for treatment and prevention of wide variety of human diseases.

1.2.3.5 Curcumin and tumor signaling

Extensive research over the last 50 years has indicated that this polyphenol can both prevent and treat cancer. Moreover, it has been demonstrated that curcumin was able to suppress tumor initiation, promotion, and metastasis. The anticancer potential of curcumin stems from its ability to suppress proliferation through modulation of various cell signaling pathways (Table 6). These may include transcription factors (nuclear factor kappa B (NF-κB) and AP1), mitogen-activated protein kinases, growth factor receptor kinases, and cyclooxygenases.

Table 6 Effect of curcumin on different cell signaling pathways (140).

Inhibition of NF-κB signaling pathway:
- Suppresses the activation of transcription factor NF-κB
- Inhibits IL-1α and TNF-induced NF-κB
- Inhibits TPA-induced activation of NF-κB
- Inhibits anticancer drug-induced activation of NF-κB
- Inhibits TNF production and release
- Inhibits inflammatory cytokine production by peripheral blood monocytes and alveolar macrophages
- Regulation of pro-inflammatory cytokine expression

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Table 6 (continued)

- Blocks NF-κB activation and proinflammatory gene expression by inhibiting IκB kinase activity - Down-regulates chemokine expression and release - Inhibits the angiogenic response stimulated by FGF-2, including expression of MMP-9 - Inhibits IL-1-stimulated NF-κB and down-regulates MMP gene expression - Inhibits TNF-mediated cell surface expression of adhesion molecules and of NF-κB activation - Reduces endothelial tissue factor gene expression - Inhibits COX2 transcription and expression - Inhibits iNOS expression and nitrite production - Induces p21 (WAF1/CIP1) and C/EBPs expression
Inhibition of AP1 signaling pathway: - Suppresses PMA-induced c-Jun/AP1 activation - Inhibits TNF-induced expression of monocyte chemoattractant JE *via* fos and jun genes - Inhibits TPA induced expression of c-fos, c-jun and c-myc proto-oncogenes mRNAs - Inhibits TPA- and UV-B light-induced expression of c-Jun and c-Fos - Reduces endothelial tissue factor gene expression - Inhibits IL1 a and TNF- induced AP1 - Inhibits TPA-induced activation of AP1 - Inhibits thrombin-induced, AP1-mediated, plasminogen activator inhibitor 1 expression - Inhibits release of MIP-1α, MIP-1s and RANTES, and AP1 - Inhibits IL-1-stimulated AP1 and down-regulates MMP gene expression

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Table 6 (continued)

Suppresses transcription factor Egr-1 **Down-regulates transactivation and gene expression of androgen receptors** **Inhibition of MAPK pathway:** - Inhibits JNK signaling pathway - Inhibits IL-1-stimulated MAP kinases and down-regulates MMP gene expression
Inhibition of growth factor pathway: - Inhibits EGF receptor kinase activity - Inhibits ligand-induced activation of EGF receptor tyrosine phosphorylation - Inhibits PTK activity of p185neu and also depletes p185neu - Inhibits PTK activity of EGF receptor and depletes the protein
Inhibition of serine protein kinase pathway: - Inhibits protein kinase C activity induced by PMA - Inhibits phosphorylase kinase - Inhibits cyclic AMP-dependent protein kinase
Others: - Inhibits LOX and COX activities - Induces GST activity - Inhibits HIV-1 and HIV-2 proteases - Inhibits PMA-induced xanthine dehydrogenase/oxidase - Modulates brain Na^+/K^+ ATPase activity - Modulates cytochrome P450 activity - Inhibits the Ca^{2+}-ATPase of sarcoplasmic reticulum - Increases the rate of accumulation of Ca^{2+}

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Table 6 (continued)

- Inhibits SERCA Ca^{2+}+ pumps - Inhibits mammalian phospholipase D activity - Inhibits of IL-12 production in LPS-activated macrophages - Blocks TGF-s1-induced uPA expression - Induces cell migration in non-tumorigenic murine colon epithelial cells through MT-MMP expression - Inhibits heme oxygenase-1 - Modulates aryl hydrocarbon receptor - Modulates P-glycoprotein in primary cultures of rat hepatocytes - Intercalates in DNA and poison Topo II isomerase - Stimulates the stress-induced expression of stress proteins

1.2.3.5.1 Curcumin suppress NF-κB activation

Members of NF-κB transcription factor family play a central role in various responses leading to host defence, activating a rapid progression of gene expression. These transcription factors are dimeric complexes composed of different members of the Rel/NF-κB family of polypeptides. This family is distinguished by the presence of a Rel homology domain of about 300 amino acids that displays a 35% to 61 % identity between various family members (184). Although NF-κB is a ubiquitous transcription factor, it plays its critical role in the cells of the immune system, where it controls the expression of various cytokines and the major histocompatibility complex genes. The inappropriate regulation of NF-κB and its dependent genes have been associated with various pathological conditions including toxic/septic shock, graft

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versus host reaction, acute inflammatory conditions, acute phase response, viral replication, radiation damage, atherosclerosis, and cancer (184, 185). Unlike other transcription factors, the NF-κB proteins and other members of the Rel family reside in the cytoplasm in an inactive state; upon activation, they are translocated to the nucleus. The nuclear translocation of Rel proteins is induced by many agents, including inflammatory cytokines (tumor necrosis factor (TNF), lymphotoxin (LT), and interleukin (IL)-1), mitogens, bacterial products, protein synthesis inhibitors, oxidative stress (H_2O_2), ultraviolet light and phorbol esters (186, 187). Upon activation of NF-κB, a large number of genes are induced, including various inflammatory cytokines (TNF, IL-1, chemokines), adhesion molecules (ICAM-1, VCAM I, ELAM-1), COX2, matrix metallopeptidase 9 (MMP-9), and nitric oxide synthase (NOS) (186, 187).

1.2.3.5.2 Curcumin Suppresses AP1

Activating protein-1 (AP1) is one such transcription factor transactivated by various tumor-promoting agents, such as phorbol ester, UV radiation, asbestos and crystalline silica (188, 189). AP1 complexes are formed by dimers of Jun proto-oncogene family members (c-Jun, JunB, and JunD) or heterodimers of the Jun family members with the Fos proto-oncogene family members (c-Fos, FosB, Fra-1, and Fra-2). AP1 binds to a specific target DNA site (also known as the TRE) in the promoters of several cellular genes and mediates immediate early gene expression involved in a diverse set of transcriptional regulation processes (188, 190). Agents that activate NF-κB also activate the transcription factor AP1. Both of these factors are regulated by the redox status of the cell. AP1 activation has been implicated in cell

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proliferation and in chemical carcinogenesis. Studies in *ex vivo* and *in vivo* models showed that the expression of various genes regulated by AP1 play important roles in the transformation from preneoplastic to neoplastic state (191). AP1 is also known to be involved in tumor progression and metastasis. *In vitro* experiments showed that inhibition of c-Jun/AP1 binding to its cognate motif by curcumin may be responsible for the inhibition of c-Jun/AP1-mediated gene expression. The expression of several AP1-regulated genes has been shown to be regulated by curcumin. These include c-fos, c-jun, c-myc, endothelial tissue factor, chemokines and MMP (140, 192-194). The mechanism by which curcumin inhibits AP1 is not fully understood, but there are three potential mechanisms: first through alteration of the redox status of the cells; second, through inhibition of JNK (195), a kinase needed for AP1 activation; and third, through inhibition of the fos-jun-DNA complex (196). Thus downregulation of AP1 by curcumin may explain its ability to suppress chemical carcinogenesis.

1.2.3.5.3 Curcumin Suppresses mitogen-activated protein kinases

Most inflammatory stimuli are known to activate three independent mitogen-activated protein kinase (MAPK) pathways, leading to activation of p44/42 MAPK (also called ERK1/ERK2), JNK, and p38 MAPK pathway. It has been reported that curcumin inhibits JNK activation induced by various agonists including PMA plus ionomycin, anisomycin, UV-C, gamma radiation, TNF and sodium orthovanadate (195). Although both JNK and ERK activation by PMA plus ionomycin were suppressed by curcumin, the JNK pathway was more sensitive. The IC_{50} of curcumin was between 5-10 μM for JNK activation and was 20 μM for ERK activation. In transfection assays, curcumin moderately suppressed MEKK1-induced JNK

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activation; however, it effectively blocked JNK activation caused by cotransfection of TAK1, GCK, or HPK1. Curcumin did not directly inhibit JNK, SEK1, MEKK1 or HPK1 activity. Although curcumin suppressed TAK1 and GCK activities at high concentrations, this inhibition cannot fully account for the JNK inhibition by curcumin *in vivo*. Thus curcumin has shown to affect the JNK pathway by interfering with the signaling molecule at the same level or proximally upstream of the MAPKKK level. The inhibition of the MEKK1-JNK pathway reveals a possible mechanism of suppression of AP1 and NF-κB signaling by curcumin and may explain the potent anti-inflammatory and anti-carcinogenic effects of this chemical (140).

1.2.3.5.4 Curcumin Suppress protein kinases

Curcumin could also mediate its effects through inhibition of various other serine/threonine protein kinases. It has been shown that treatment of highly purified protein kinase A (PKA), protein kinase C (PKC), protamine kinase (cPK), phosphorylase kinase (PhK), autophosphorylation- activated protein kinase (AK), and pp60c-src tyrosine kinase with curcumin inhibited all kinases (140). PhK was completely inhibited at low concentration of curcumin (197). Curcumin at 0.1 mM, PhK, pp60c-src, PKC, PKA, AK, and cPK were inhibited by 98%, 40%, 15%, 10%, 11%, and 0.5%, respectively. Lineweaver-Burk plot analysis indicated that curcumin is a noncompetitive inhibitor of PhK with a Ki of 0.075 mM. Several studies have shown suppression of PMA induced activation of cellular PKC by curcumin (198). Treatment of cells with 15 or 20 μM curcumin inhibited TPA induced PKC activity in the particulate fraction by 26% or 60%, respectively, and did not affect the level of PKC. Curcumin also inhibited PKC activity in both cytosolic and particulate

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fractions *in vitro* by competing with phosphatidylserine. However, the inhibitory effect of curcumin was reduced after preincubation with the thiol compounds. These findings suggested that the suppression of PKC activity may contribute to the molecular mechanism of inhibition of TPA induced tumor promotion by curcumin. *In vitro* suppression, curcumin could also inhibit PKC in the cells (199). The curcumin inhibits Ca^{2+}-and phospholipid-dependent PKC and of the catalytic subunit of cyclic AMP-dependent protein kinase (cAK; IC_{50} values of 15 and 4.8 μM, respectively) (199). Curcumin inhibits plant Ca^{2+}-dependent protein kinase (CDPK) (IC_{50} value of 41 μM), but does not inhibit myosin light chain kinase or a high-affinity 3',5'-cyclic AMP-binding phosphatase. Curcumin also inhibits cAK, PKC and CDPK in a fashion that is competitive with respect to both ATP and the synthetic peptide substrate employed. The IC_{50} values for inhibition of cAK by curcumin are very similar when measured with kemptide (LRRASLG) (in the presence or absence of ovalbumin) or with casein or histone III-S as substrates. However, the presence of bovine serum albumin (0.8 mg/mL) largely overcomes inhibition of cAK by curcumin.

1.2.3.5.5 Curcumin Suppresses Egr-1

The transcription factor early growth response-1 gene product (Egr-1) is a member of the family of immediate early response genes and regulates a number of patho-physiologically relevant genes in vasculature. It is involved in growth, differentiation, immune response, wound healing and blood clotting. Pendurthi *et al.* (2000) investigated the effect of curcumin on Egr-1 expression in endothelial cells and fibroblasts (200). Gel mobility shift assays showed that pretreatment of

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endothelial cells and fibroblasts with curcumin suppressed TPA and serum-induced Egr-1 binding to the consensus Egr-1 binding site and also to the Egr-1 binding site present in the promoter of the tissue factor gene. Western blot analysis revealed that curcumin inhibited TPA-induced *de novo* synthesis of Egr-1 protein in endothelial cells. Suppression of Egr-1 protein expression in curcumin-treated cells stemmed from the suppression of Egr-1 mRNA. Northern blot analysis showed that curcumin inhibited serum and TPA-induced expression of tissue factor and urokinase-type plasminogen activator receptor mRNA in fibroblasts.

1.2.3.5.6 Curcumin inhibits growth factor receptor protein tyrosine kinases

Besides serine protein kinases, curcumin has also been shown to inhibit protein tyrosine kinase activity (PTK) of the EGF receptor (201-203). Korutla *et al.* (1994) showed that the short term treatment of cells with curcumin inhibited EGF receptor intrinsic kinase activity by up to 90%, and also inhibited EGF-induced tyrosine phosphorylation of EGF receptors (201). Another study found that the treatment of cells with a saturating concentration of EGF for 5-15 min induced increased EGF-R tyrosine phosphorylation, and this induction was inhibited by up to 90% by curcumin, which also inhibited the growth of EGF-stimulated cells (202). Curcumin treatment had no effect on the amount of surface expression of labeled EGF-R, and inhibition of EGF-mediated tyrosine phosphorylation of EGF-R by curcumin was mediated by a reversible mechanism. In addition, curcumin also inhibited EGF-induced, but not bradykinin-induced, calcium release. These findings demonstrate that curcumin is a potent inhibitor of a growth stimulatory pathway, the ligand-induced activation of EGF-R, and may be useful in developing

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antiproliferative strategies to control tumor cell growth. The ErbB2/neu gene-encoded p185neu tyrosine kinase is a potent oncoprotein. Overexpression of p185neu in breast cancer is known as a prognostic factor. Hong *et al.* (1999) investigated the effect of curcumin on p185neu tyrosine kinase and on the growth of breast cancer cell lines (204). Curcumin inhibited p185neu autophosphorylation and transphosphorylation *in vitro* and depleted p185neu protein *in vivo.* It dissociated the binding of p185neu with GRP94 (glucose-regulated protein), a molecular chaperone, and enhanced the depletion of p185neu. The amount of p185neu protein on the cell membrane was drastically decreased after curcumin treatment. Moreover, the growth of several breast cancer cell lines was inhibited; the IC_{50} ranged from 7 to 18 μM, which, however, did not correlate with the expression level of p185neu. Colony formation in the soft agar assay, a hallmark of the transformation phenotype, was preferentially suppressed in p185neu-overexpressing cell lines by 5 μM curcumin. Because curcumin effectively inhibited p185neu tyrosine kinase activity by depleting p185neu and potently suppressed the growth of multiple breast cancer cell lines, its therapeutic potential in advanced breast cancer is worthy of further investigation. Furthermore, curcumin was a potent inhibitor of EGF-R signaling, and it accomplished this effect by three different means: (1) down-regulating the EGF-R protein; (2) inhibiting the intrinsic EGF-R tyrosine kinase activity; and (3) inhibiting the ligand-induced activation of the EGF-R (145).

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Thus, curcumin plays a major role in its ability to prevent cancers. It could down-regulate TNF-induced NF-κB-regulated gene products involved in cellular proliferation (COX-2, cyclin D1, and c-myc), antiapoptosis (IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, Bf1-1/A1, TRAF1, cFLIP) and metastasis (VEGF, MMP-9, ICAM-1).

Moreover, curcumin can inhibit IL-6-induced STAT3 phosphorylation and conquest STAT3 nuclear translocation. Curcumin had no effect on STAT5 phosphorylation however it inhibited interferon-α-induced STAT1 phosphorylation. These studies suggest that curcumin inhibited cancer development and progression by targeting multiple steps in the pathway to malignancy (Figure 7). Curcumin has an enormous potential in prevention of the mechanism would enhance the therapeutic potential of curcumin either alone or combination with chemotherapy.

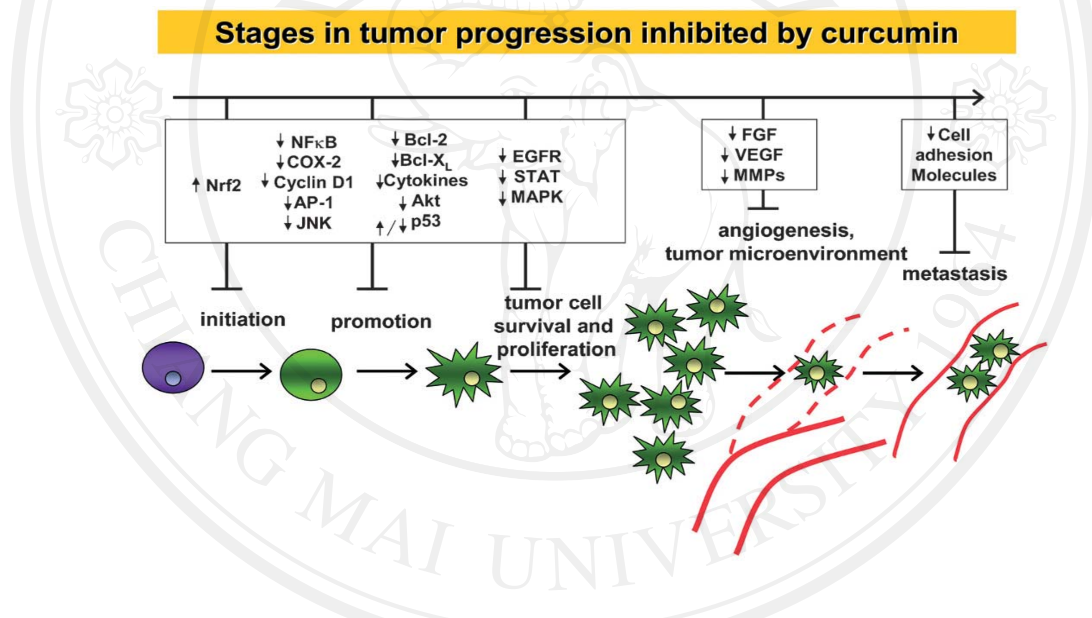

Figure 7 Stages in tumor progression inhibited by curcumin (141)

1.2.3.6 Curcumin and leukemia

Study of curcumin *in vitro*, Curcumin has been shown to have synergistic and medical properties in leukemia. The effect of curcumin at 10 μM can decrease proliferation and increase differentiation cell in HL-60 cell line. Furthermore, these effects were exacerbated when curcumin was given in conjunction with RA, vitamin D3 and vitamin D3 analogs (205-208). However, curcumin alone caused a significant

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reduction in NF-κB expression, Bcl-2 activity, and TPA-induced DNA binding. Moreover, it can induce ER stress bax and caspases 3 and 8 and degrades poly ADP ribose polymerase (PARP) (182, 208, 209). Its mechanism involves the interruption of G0/G1 phases associated with the up-regulation of P27kipl, P21walf, and pRbp-expression and the down-regulation of cyclin D3 (182, 210). In HL-60 cells, curcumin and its analog, alpha-diisoeugenol can induce ROS levels while curcumin alone up-regulated Ca^{2+} production and the release of cytochrome c and lowered MMP levels (211-213). When coupled with TSA, curcumin increased histone acetylation, increasing cytotoxicity for HL-60 cells (212). In Raji cells, curcumin selectively blocks tumor cells in the G0/G1 and G2/M phases; dose-dependently upregulates Ac-histone H4 expression which inhibit the proferation and degradation of IκBα and Notch 1; and inhibits the translocation of the NF-κB/p-65 subunit *via* the downmodulation of HDAC1 and p300/Notch 1 signal molecules (214-217). Similarly, in K562 cells that were treated with curcumin, telomerase was translocated which causing a loss of telomerase activity and expression of STA3, -5a and 5b are reduced without altering STAT1 or the phosphorylation states of STA1,-3 or -5 *via* the release of cytochrome c release from mitochondria (218, 219). Beside, curcumin also affects GST-modulated lipid peroxidise, activation protein-1 (AP1) and NF-κB binding to GSTP1-1 promoters, PARP cleavage and pro-caspases 8 and 9 induction in K562 cells (9, 220). Furthermore, down regulation of both JAK and STAT phosphorylation are dose dependent of curcumin which is alter cause of growth inhibition and apoptosis by inhibiting cyclin D1, cdk1 Cdc25C, and XIAP and Survivin expression in T cell leukemia, HTLV-I-transformed T cell leukemia, MT-2, HuT-102, and SLB-1 cell lines (221, 222). In CML cells, curcumin suppresses the

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proliferation of WEHI-3B cells and blocks STAT5 mRNA expression and STAT5 activation (223, 224). While TK-10 and UACC-62 cell lines, curcumin can initiate apoptosis via telomerase II poisoning which causes DNA damage (225). Curcumin can induce increased nitric oxide (NO) levels in acute leukemia cells which were exposed to curcumin for 4 h (226). The increasing of NO production by macrophages and the inhibition of Th1 cytokines in NK cells in the presence of curcumin lead to significant tumoricidal results (227). The proliferation of Jurkat cells was reduced when treated with curcumin, resulting in chromatin condensation and capase-3 induction via the prevention in a decrease of glutathione levels (228, 229). In Bcr-Abl-transfected mouse progenitor 32D cells, curcumin inhibits proliferation by arresting cells in the G2/M phase of the cell cycle, resulting in irregular chromatin organization, multipolar chromosome segregation, aberrant cytoknesis, and multinucleated cell with morphologic changes (230). Moreover, curcumin analogs in KBM-5 cell blocked TNF-induced NF-κB activation and proliferation and curcuminoid inhibited COX-I and COX-II enzyme (231, 232).

Studies have also demonstrated curcumin's therapeutic properties *in vivo*. In 6-week-old mice, the administration of a 2% curcumin diet *via* oral gavage resulted in a 53% reduction in lymphoma and leukemia. When topically applied prior to the administration of TPA in mice, curcumin down-regulated TPA-induced NF-κB and AP1. It also showed that oral administration of curcumin (50-200 mg/kg) inhibited the development of leukemia (HL-60) cells induced xenografts in nude mice (182). In a group of 10 male smokers, 10 male non-smokers, and 10 female non-smokers women between 25 and 45 years of age, curcumin reduced BP-stimulated strand breaks in a

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sex-dependent manner (233). In 70 samples of childhood leukemia patients, curcumin reduced *WT1* gene expression in 35 samples (7).

1.2.3.7 Curcumin in human clinical trials

Curcumin is under active investigation for its clinical benefit, although clinical trials are still in relatively early phases. Promising initial results were reported in limited subsets of patients treated with curcumin for chronic anterior uveitis (234), idiopathic inflammatory orbital pseudo tumors (235), post-operative inflammation (236), external cancerous lesions (237) and pancreatic cancer. Early trials emphasized safety and pharmacokinetics. While continuing to assess these aspects of curcumin's activity, current trials are also exploring efficacy. Consonant with preclinical demonstrations of curcumin's anti-inflammatory and anti-cancer properties, disease targets include neoplastic and preneoplastic diseases such as multiple myeloma, pancreatic cancer, myelodysplastic syndromes, and colon cancer (178, 238), and conditions linked to inflammation such as psoriasis, and Alzheimer's disease (Table 7).

Table 7 Clinical trial of curcumin (141)

Trial	Status of trial	Site	Disease target	Objective
Curcumin with or without bioperine	Ongoing	MD Anderson, USA	Multiple myeloma	Tolerance and safety of curcumin vs curcumin plus bioperine

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Table 7 (continued)

Pharmacokinetics of curcumin in healthy volunteers	Ongoing	MGH, USA	None	Curcumin pharmacology with piperine or silybin
Gemcitabine with curcumin for pancreatic cancer	Ongoing	Rambam Medical Center Haifa Israel	Pancreatic cancer	Clinical benefit of gemcitabine plus curcumin in pancreatic cancer Phase II trial
Trial of curcumin in advanced pancreatic cancer	Ongoing	MD Anderson, USA	Pancreatic cancer	Response rate and pharmacokinetics in pancreatic cancer, Phase II trial
Efficacy of coenzyme Q10 and curcumin in patients with MDS	Not yet open	Hasdassah Medical Organization, Jerusalem Israel	Myelodysplasia	Hematological improvement in patients with MDS
Curcumin in patiens with mild to moderate Alzheimer's disease	Ongoing	UCLA medical center, USA	Alzheimer's disease	Safety, biodistribution, efficacy

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Table 7 (continued)

Phase III trial of gemcitabine, curcumin and celebrex in patients with metastaic colon cancer	Not yet open	Tel-Aviv Sourasky Medical Canter, Israel	Colon cancer	Efficacy (time to progression) Phase III
Curcumin in preventing colon cancer in smokers with ACF	Ongoing	Multicenter, USA (meyskens PI)	Colon cancer	Preventio-change in prostaglandin E2 in ACF
Use of curcumin in the familial adenomatous polyposis patients	Ongoing	Johns Hopkins, USA	Colon cancer	Regression of colorectal adenomatous polyps in patients with familial adenomatous polyposis
Curcuminoids for the treatment of chronic psoriasis vulgaris	Ongoing	University of Pennsylvania, USA	Psoriasis	Safety, efficacy

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Table 7 (continued)

Curcumin for the chemoprevention of colorectal cancer	Ongoing	University of Pennsylvania, USA	Colon cancer	Prevention-effect on cell proliferation, apoptosis and COX2 in the colonic mucosa of patients with sporadic adenmatous polyps
Effect of curcumoids on ACF in the human colon	Ongoing	University of Medicine and Dentistry New Jersey USA	Colon	Prevention-effect of curcumin or sulindac on number of ACF in colon
Pilot study of curcumin and ginkgo for treating Alzheimer's disease	Closed	Chinese University of Hong Kong	Alzheimer's disease	Effect on ioprostanes, amyloid beta protein, cognitive function
Curcumin for the prevention of colon cancer	Closed (completed)	University of Michigan	None	Phamacokinetics, MTD, phase I trial in healthy subjects

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Table 7 (continued)

Sulindac and plant compound in preventing colon cancer	Closed (completed)	Rockefeller University	Colon cancer	Prevention-effect of curcumin on biomarkers of colon epithelial cell turnover
Phase I Trial in patients with premalignant lesions	Completed	National Taiwan University college of medicine	Various	Pharmacokintics and MTD
Phase I trial: biomakers	Completed	University of Leicester and University of Liverpool, UK	Colorectal cancer	Pharmacokinetics, MTD, biomarkers
Phase III trial of gemcitabine curcumin and celebrex in patients with advance or in operable pancreatic cancer	Ongoing	Tel-Aviv Sourasky Medical Canter, Israel	Pancreatic cancer	Cilincal benefit of gemcitabine plus curcumin and Celebrex in pancreatic cancer, Phase III

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Table 7 (continued)

Bio-availability of a new liquid turmeric extract	Not yet open	Hadassah Medical Orgaization, Jerusalem, Israel	Healthy	Pharmacokinetics
Epilepsy	-	AIIMS, Delhi, India	Epilepsy	Phase I
Advanced	-	Himalayan Institute of Medical Sciences	Advanced HNSCC	Phase II (1-8g/day; 56 days)
HNSCC	-	AIIMS, Delhi, India	HNSCC	Phase II/III DBRPC (3.6 g/day, bid)
Cervical cancer (Stage IIb, IIIb)	-	AIIMS, Delhi, India	Oral cancer	Phase II/III DBRPC (2 g/day, bid, 1 years)
Oral premalignant lesions	-	Tata Memorial Cancer Center, India	Oral cancer	Phase II/III DBRPC (4g/day, bid x28 days)
Oral premalignant lesions	-	Regional Cancer Center, Thriven, india	Oral leukoplakia	Phase II (curcumin gel, 3 x/day, 6 months)

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Table 7 (continued)

Gall bladder cancer	-	BHU, India	Gall bladder cancer	Phase II (2-8 g/day)
Pancreatic cancer	Ongoing	Kyoto University, Japan	Pancreatic cancer	Phase II (8 g/day)
Primary sclerosing cholangitis	Ongoing	Amsterdam Medical Center, The Netherlands	Primary sclerosing cholangitis	Phase I (8 g/day)
Ulcerative colitis	Ongoing	Amsterdam Medical Center, The Netherlands	Ulcerative colitis	Phase I (8 g/day)
Barretts Metaplasia	Ongoing	Amsterdam Medical Center, The Netherlands	Barretts Metaplasia MGUS	
Monoclonal gammopathy of unknown significance	Ongoing	St.George Hospital, Australia	MGUS	Phase I (3.4 g/day)

Serum curcumin concentration peaked 1–2 h after oral intake and then gradually declined. Maximum serum concentration ranged from 0.5±0.11 mM at 4,000 mg/day

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to 1.77±1.87 mM at 8,000 mg/day. At lower doses, curcumin was not detectable in serum. Pharmacokinetic parameters remained the same after patients had taken curcumin for 1 month. Curcumin was not detected in the urine. Although it was not the primary objective of the study, histological examination of precancerous lesions following curcumin treatment revealed improvement in some cases, including 1 patient with bladder cancer, 2 patients with intestinal metaplasia of the stomach, 1 patient with CIN and 2 patients with Bowen's disease. An independent dose-escalation study on 15 patients with advanced colorectal cancer was conducted in the UK (239). Patients consumed a single daily dose of 440-2,200 mg curcuma extract, equivalent to 36-180 mg curcumin, for up to 4 months. The treatment was well tolerated and there was no dose-limiting toxicity. Consistent with results reported by Cheng *et al*. (2001) neither curcumin nor its metabolites were detected in the plasma, blood cells or blood lipoproteins at up to 29 days of daily treatment (177). Curcumin was not detected in the urine, but both curcumin and curcumin sulfate were present in feces. Stable disease was observed in 5 patients receiving 2-4 months of therapy. Blood from patients in this trial was also used to explore the utility of leukocyte COX-2 as a biomarker for curcumin; however, measurements of blood levels of PGE2, a product of COX-2, were not significantly different in subjects who did and did not consume curcumin (240). Studies in healthy human volunteers consuming a single dose of curcumin ranging from 500 to 12,000 mg gave a similar overall picture (178). Non dose-limiting toxicities were observed, and low levels of curcumin were only detected in the serum receiving the highest doses of curcumin (10,000 or 12,000 mg/day). Interestingly, curcumin was only detected in 2 of these 6 patients, perhaps indicating the existence of genetic modifiers of curcumin metabolism. These authors

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also discovered a greater than twofold variation in the curcumin content of different preparations of commercially procured curcumin, which may partially account for low serum levels despite apparently high consumption. This point to one of the difficulties associated with interpreting the literature on curcumin, which is the infrequency with which curcumin content is measured and reported. Curcumin is particularly abundant in *Curcuma longa* (3.9-12.3%), but curcumin and curcuminoids have also been isolated from a variety of other plant species, including *Curcuma aromatica* (0.11%), and *Curcuma phaeocalis* (0.89%) (241). Curcumin content varies among the many commercially available blends of turmeric and curry powders (242). For example, one study estimated the percentage of curcumin to be between 1.06% and 5.70% in four different "commercially available" turmeric samples (243). Pure turmeric was found to have the highest concentration of curcumin with an average of 3.14% by weight, while curry powders contained relatively low amounts of curcumin (141). In addition, curcumin itself exists in several forms that exhibit different potencies as antioxidants and anti-tumor agents (160, 244). Thus, the actual amount of curcumin used in various studies is often unclear. Curcumin has also been measured in human tissue, example, in the liver and portal blood of 12 patients undergoing resection of hepatic metastases of colorectal cancer who took 450-3,600 mg curcumin daily for 1 week prior to surgery (245). Low nanomolar levels of curcumin and its metabolites, curcumin glucuronide and curcumin sulfate, were detected in portal serum of all 3 patients who received 3,600 mg of curcumin. Metabolic reduction products of curcumin (hexahydrocurcumin and hexahydrocurcuminol) were found in the liver of 1 patient. The authors concluded that the bioavailability of curcumin is poor in tissues remote from the gastrointestinal tract, including the liver.

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1.2.3.8 Safety and pharmacology

1.2.3.8.1 Pharamacokinetic of curcumin

The absorption, metabolism and tissue distribution of curcumin has been studied in at least 10 studies performed in rodents over the past three decades. In an early study, a dose of 1 g/kg was administered to rats in the diet (246). About 75% of the dose was excreted in the faeces and negligible amounts appeared in the urine. A few years later, a study of oral curcumin administered to rats demonstrated 60% absorption of curcumin and presented evidence for the presence of glucuronide and sulphate conjugates in urine (247). Indeed, the bioavailability of curcumin using ^{3}H-radiolabelling; oral administration resulted in the vast majority of the oral dose being excreted in faeces, and only one-third was excreted unchanged (248). Intravenous and intraperitoneal administration of curcumin in rodents resulted in large quantities of curcumin and metabolites in bile, which were characterised as mainly tetrahydrocurcumin and hexahydrocurcumin glucuronides (158, 249). After intravenous dosing, more than 50% of the dose was excreted in the bile within 5 h; these data were presented as evidence that curcumin undergoes transformation during absorption *via* the intestine and is possibly subject to entero-hepatic recirculation (249). Such an hypothesis was originally presented by Holder, Plummer and Ryan (158) based on their studies of the fate of curcumin in rats. A more recent study of intraperitoneal curcumin (0.1 g/kg) in the mouse has suggested that curcumin was first biotransformed to dihydrocurcumin and tetrahydrocurcumin, and that these compounds were subsequently converted to monoglucuronide conjugates (250). Preclinical studies of oral dosing of curcumin in rats using modern high pressure liquid chromatography (HPLC) techniques demonstrate small amounts of curcumin in

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plasma with higher levels of curcumin glucuronide and curcumin sulphate in plasma, and small quantities of hexahydrocurcumin, hexahydrocurcuminol and hexahydrocurcumin glucuronide (Figure 8) (156, 251). This preclinical work was extended using suspensions of isolated human hepatocytes or liver or gut microsomes (157). The data suggested that metabolic reduction occurs very rapidly, in a matter of min. A study of high dose curcumin (2% in the diet, equating to approximately 1.2 g curcumin per kg body weight) for 14 days has shown that low nanomolar levels are detectable in plasma, with concentrations in liver and colon mucosal tissue ranging from 0.1 to 1.8 nmol/g tissue (252). In a study of oral curcumin (2 g/kg) in rats performed in Bangalore, India, the investigators suggested that co-administration of piperine may increase systemic bioavailability following oral dosing by as much as 154%, potentially by inhibition of xenobiotic glucuronidation (181). Piperine is primarily found in the fruit of the pepper vine, piper nigrum, and can also be found in other vegetables and spices such as hot jalapeno peppers. It is also said to give peppercorns their hot, biting and pungent taste.

1.2.3.8.2 Safety

Curcumin is remarkably well tolerated, but its bioavailability is poor. It does not appear to be toxic to animals (180) or humans (253) even at high doses. Cheng *et al.* (2001) conducted a Phase I trial of curcumin in patients with high risk or premalignant lesions in Taiwan; 24 patients completed the study (177). Patients included those with resected bladder cancer, oral leukoplakia, stomach metaplasian, cervical intraepithelial neoplasm (CIN) and Bowen's disease. Curcumin was administered as a single daily oral dose ranging from 500 to 8,000 mg/day for 3 months.

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Non toxicity was observed at any dose. A planned escalation to 12,000 mg/day was not carried out since the bulky volume of the tablets was not acceptable to patients. Pharmacokinetic studies were performed in patients receiving 4,000-8,000 mg/day.

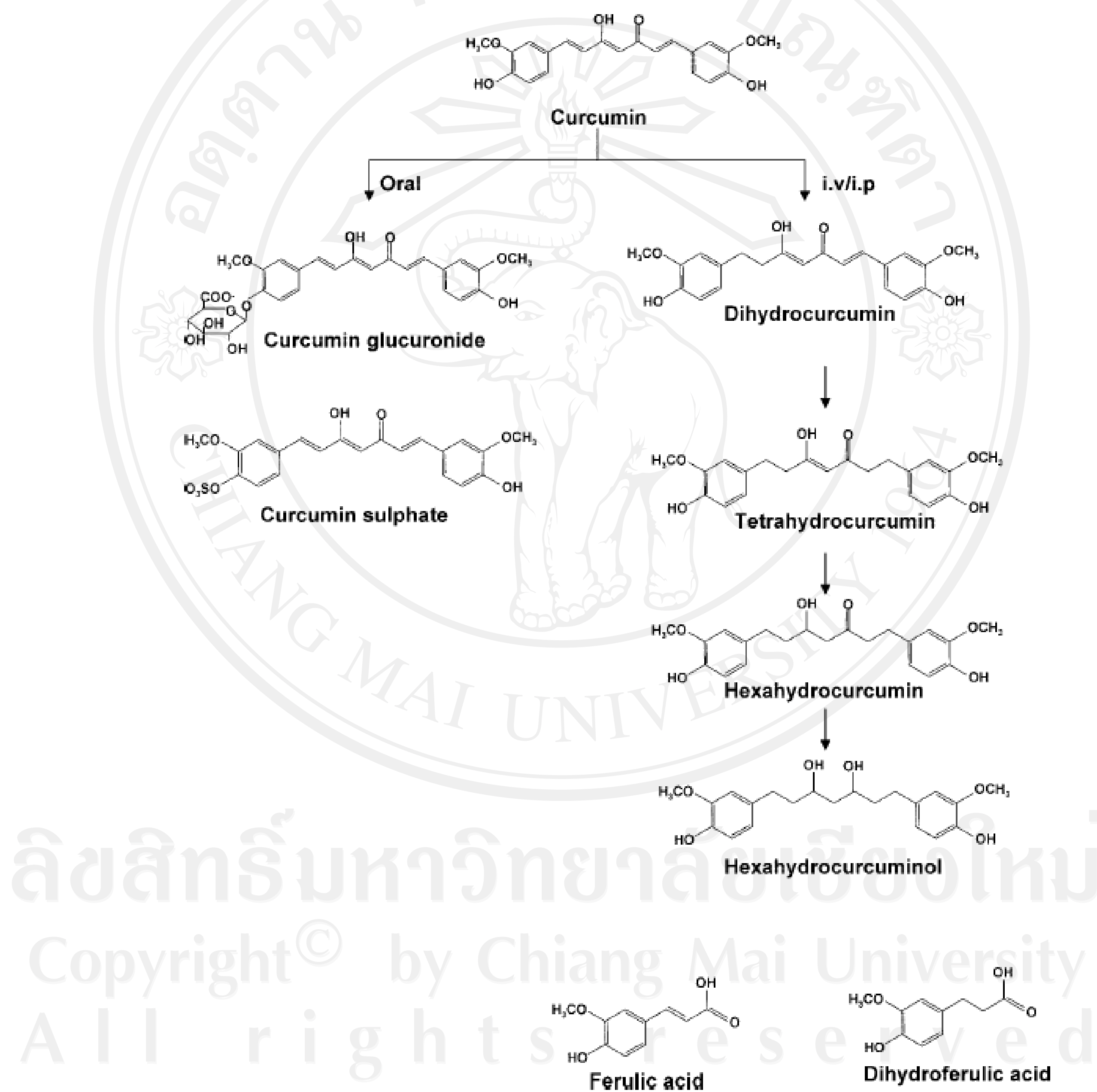

Figure 8 Structure of curcumin and its metabolite (251)

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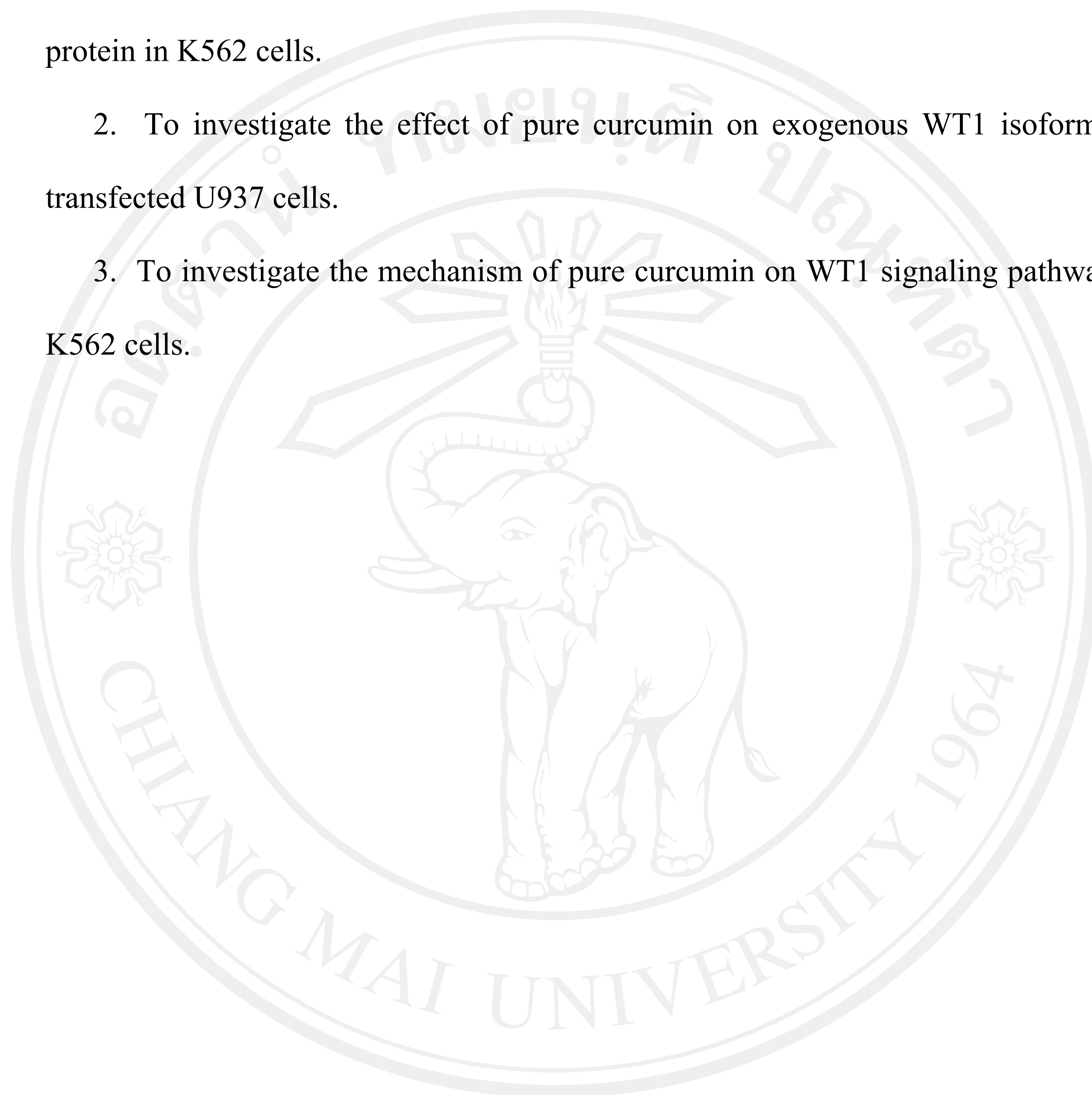
1.3 Objectives

1. To investigate the effect of pure curcumin on endogenous *WT1* gene and WT1 protein in K562 cells.

2. To investigate the effect of pure curcumin on exogenous WT1 isoforms in transfected U937 cells.

3. To investigate the mechanism of pure curcumin on WT1 signaling pathway in K562 cells.



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