Chapter 1

Introduction
1.1 **Haematopoiesis**

Haematopoietic system consists of various cell types with defined functions. Red blood cells (erythrocytes) carry oxygen to the tissues, platelets help to prevent bleeding, granulocytes (neutrophils, basophils and eosinophils) and macrophages (collectively known as myeloid cells) fight infections from bacteria, fungi and other parasites such as nematodes. B-lymphocytes produce antibodies while T-lymphocytes can directly kill or isolate them by inflammation that are recognized as foreign to the body which includes virus-infected cells and cancer cells. Many blood cells are short-lived and need to be replenished continuously; the average human requires approximately one hundred billion new haematopoietic cells each day. The continued production of these cells depends directly on the presence of Haematopoietic Stem Cells (HSCs). The process by which HSC commit and differentiate to all blood lineage cells is defined as haematopoiesis.

Haematopoiesis is the process of formation of cellular components of blood. Haematopoietic stem cells give rise to the cellular components which reside as rare cells in the bone marrow in adult mammals and sit atop a hierarchy of progenitors that become progressively restricted to several or single lineages (1). More than $2 \times 10^{11}$ haematopoietic cells from at least 11 lineages are produced daily in humans from a small pool of self-renewing adult stem cells (2). Production of each cell type is highly regulated and responsive to environmental stimuli. Due to stress and mutations several haematological disorders can occur. A family tree of blood cell lineage with the transcription factors required for the haematopoiesis is depicted in Figure 1. Haematopoietic stem cells yield blood precursors devoted to uni-lineage differentiation and production of mature blood cells, including red blood cells, megakaryocytes, myeloid cells (monocytes/macrophages and neutrophils), and lymphocytes (3-6). HSCs are capable of self-renewal—the production of additional HSCs—and differentiation, specifically to all blood cell lineages. In mammals, the sequential sites of haematopoiesis include the yolk sac, aorta-gonad-mesonephros (AGM); a region of embryonic mesoderm, the fetal liver, and the bone marrow (7-12).
Figure 1. Haematopoiesis and transcription factors required in the process. The stages at which haematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts, are indicated by red bars. The factors depicted in black have been associated with oncogenesis. Those factors in light font have not yet been found translocated or mutated in human/mouse haematologic malignancies. Abbreviations: LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocytes/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells (adapted from Orkin et al. Cell, 2008(13)).

Recently placenta has also been recognized as an additional site (14). Stem cell development depends on the microenvironment, the niche for regulation of self renewal and differentiation (15).
1.1.1 Haematopoietic Lineage Differentiation

The haematopoietic lineage differentiation is a hierarchical fashion, haematopoietic stem cells first produce the progenitor cells and then the precursors with varying commitments to multiple or single pathways are formed. After a transient harboring in the yolk sac and AGM, HSCs migrate to the secondary sites of haematopoiesis: the liver, spleen, thymus and bone marrow, where the totipotent HSCs give rise to a population with restricted differentiation capacity (16, 17). These progenitor cells which can be identified by expression of specific lineage markers and bioassays of developmental potential in tissue culture or transplantation eventually differentiate into different haematopoietic lineages. The common lymphoid progenitors (CLP) give rise to B-cells and T-cells (18), whereas the common myeloid progenitors (CMP) give rise to monocytes, platelets, granulocytes and erythrocytes (19). The developmental potential of these cells is generally limited to only one or two of the haematopoietic lineages and these cells progressively display the antigenic, biochemical, and morphological characteristic of the mature cells of the appropriate lineages and lose their capacity for self-renewal.

1.1.2 Molecular Regulation of Haematopoiesis

Haematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and the environment surrounding it. This interplay determines whether HSCs, progenitors and mature blood cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis. The best characterized environmental regulators of haematopoiesis are cytokines (20). Cytokines are a broad family of proteins that mediate positive and negative effects on cellular quiescence, apoptosis, proliferation, and differentiation. Cytokines function through a specific receptor and activate a variety of signaling pathways. This includes activation of tyrosine kinases such as focal adhesion kinase, pp60src, c-Abl, MAP kinases, Jun N-Terminal Kinase (JNK), and protein kinase C (PKC) (21). Cytokines including interleukin-3 and GM-CSF induce cell proliferation, while other cytokines including flt-3 ligand and kit ligand protect cells from apoptosis and sensitize them to the effects of growth promoting cytokines (22, 23) Cytokines may also facilitate the interactions between stem cells and elements in the microenvironment including extracellular matrix (ECM) components (24). Haematopoietic regulatory cytokines are
produced through both autocrine and paracrine mechanisms and in many cases are produced by non-haematopoietic cells including bone marrow stroma and endothelium.

Chemokines are another class of compounds that are important regulators of haematopoiesis (25). Chemokines are composed of a large family of proteins that mediate a variety of processes including inflammation, leukocyte migration and development, angiogenesis, tumor cell growth and metastasis (26). Chemokines bind to one or more of a large family of structurally related guanine protein-coupled transmembrane receptors (GPCRs). In haematopoiesis, chemokines can inhibit progenitor growth, regulate migration of haematopoietic progenitors, and mediate T-cell development in the thymus (27). Chemokine SDF-1 (which binds the receptor CXCR4) is essential for trafficking of haematopoietic cells in the developing embryo (26). A number of other chemokines likely play important roles in haematopoiesis. Other important environmental regulators of haematopoiesis include the extracellular matrix (ECM) components, other haematopoietic and nonhaematopoietic cells, nutrients and vitamins, and a variety of physiologic processes.

The haematopoiesis program is controlled by multiple factors and pathways. Gene silencing studies have shown effects of various genes on the haematopoietic program. T-lymphoid acute leukemia oncoprotein Tal-1/SCL (28-31) and LMO2 knockout mice (32) lack the precursor determination or maintenance both in yolk-sac and fetal liver which indicates that these two genes play important roles in early development of primitive and definitive haematopoietic progenitors. Although no specific primitive haematopoietic deficient mutant has been identified so far, studies from these knock-out mice indicate that the requirements for primitive and definitive haematopoiesis are distinct. Similarly, absence of the erythroid cell in GATA1 knock-out mice (33) and lymphoid cells in Ikaros knock-out mice (34, 35) demonstrated that GATA1 and Ikaros are key regulators in erythropoiesis and lymphopoiesis respectively.

1.1.3 **Properties of Haematopoietic Stem Cells**

The core property of haematopoietic stem cells is the ability to choose between self-renewal (remain a stem cell after cell division) and differentiation (start the path towards becoming a mature haematopoietic cell). In addition, HSCs migrate in regulated manner during development.
process and are subject to regulation by programmed cell death (apoptosis). The balance between these activities determines the number of stem cells in the body.

1.1.3.1 Self-Renewal

One essential feature of HSCs is the ability to self renew i.e. to make copies with the same or very similar potential. This is an essential property because more differentiated cells, such as haematopoietic progenitors, cannot do this, even though they can expand significantly during a limited period of time after being generated. The key components or signals which allow self renewal are still unknown but one noteworthy key link is telomerase, the enzyme necessary for maintaining telomeres, the DNA regions at the end of chromosomes that protect them from accumulating damage due to DNA replication (36). Expression of telomerase is associated with self-renewal activity (37, 38). However, while absence of telomerase reduces the self renewal capacity of mouse HSCs, forced expression is not sufficient to enable HSCs to be transplanted indefinitely; other barriers must exist. It has proven surprisingly difficult to grow HSCs in culture despite their ability to self-renew.

1.1.3.2 Differentiation

The property of HSCs to differentiate into progenitors and mature cells that fulfill the functions performed by the haematopoietic system along with the self renewal property, defines the core function of HSCs. Differentiation is driven and guided by an intricate network of growth factors and cytokines. Once the HSCs commit to differentiate they cannot revert to a self-renewing state. Thus, specific signals, provided by specific factors seem to be required to maintain HSCs. This strict regulation reflects the proliferative potential present in HSCs, deregulation of which could easily result in malignant diseases such as leukemia or lymphoma.

1.1.3.3 Migration

Migration is another important property of HSCs which occurs at specific times during development (i.e., seeding of fetal liver, spleen and eventually, bone marrow) and under certain conditions (e.g., cytokine-induced mobilization). These migrating cells enter empty haematopoietic niches in the bone marrow and provide sustained haematopoietic stem cell self-
renewal and haematopoiesis (39, 40). It is assumed that this property of mobilization of HSCs is highly conserved along the evolution (mouse, dog and humans) and presumably results from contact with natural cell-killing agents in the environment after which regeneration of haematopoiesis requires restoring empty HSC niches. This means that functional, transplantable HSCs course through every tissue of the body in large numbers every day in normal individuals.

1.2 Leukemia

Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells. Cancer cell growth is different from normal cells; instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells can also invade other tissues something that normal cells cannot.

Leukemias are proliferative diseases of the hematopoietic system that fail to obey normal regulatory signals. They are derived from stem cells or progenitors of the hematopoietic system and almost certainly include several stages of progression. During this progression, genetic and/or epigenetic changes occur; either in the DNA sequences itself (genetic) or other heritable modifications that affect the genome (epigenetic). These epigenetic changes alter cells from the normal hematopoietic system into cells capable of robust leukemic growth. There are varieties of leukemia, usually classified by the predominant pathologic cell types and/or the clinical course of the disease.

1.2.1 Types of leukemia

Based on cell type and phenotype of blast cells, leukemia can be divided into 4 main types:

- Acute myeloid (or myelogenous) leukemia (AML)
- Chronic myeloid (or myelogenous) leukemia (CML)
- Acute lymphocytic (or lymphoblastic) leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)
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Acute leukemia versus Chronic leukemia

The first factor in classifying a patient's leukemia is whether most of the abnormal cells are mature (look like normal white blood cells) or immature (look more like stem cells). In acute leukemia, the bone marrow cells do not mature properly and these immature leukemia cells continue to reproduce and build up. Without treatment, most patients with acute leukemia would live only few months. Some types of acute leukemia respond well to treatment and many patients can be cured while other types of acute leukemia have a less favorable outlook. In chronic leukemia, the cells can mature partly but not completely. These cells may look fairly normal but they are not. Unlike normal white blood cells, they do not fight infections; rather they survive longer, build up and crowd out normal cells. Chronic leukemias tend to progress over a longer period of time, and most patients can live for many years. But chronic leukemias are generally harder to cure than acute leukemias.

Myeloid leukemia versus lymphocytic leukemia

The second factor in classifying leukemia is the type of bone marrow cells that are affected. Leukemias that start in early forms of myeloid cells i.e. white blood cells (other than lymphocytes), red blood cells, or platelet-making cells (megakaryocytes) are myeloid leukemias (also known as myelocytic, myelogenous, or non-lymphocytic leukemias). If the cancer starts in early forms of lymphocytes, it is called lymphocytic leukemia (also known as lymphoid or lymphoblastic leukemia). Lymphomas are also cancers that start in lymphocytes. But whereas lymphocytic leukemias develop from cells in the bone marrow, lymphomas develop from cells in lymph nodes or other organs.

1.2.2 Acute myeloid leukemia

AML is a heterogeneous malignant hematopoietic disorder characterized by accumulation of undifferentiated cells due to mutations that prevent their normal differentiation and allow undifferentiated cells to survive. Common symptoms of untreated AML are fatigue, bleeding due to thrombocytopenia, organ infiltration and fatal infections due to neutropenia, all resulting from the suppression of normal bone marrow function. AML is diagnosed on the basis of the accumulation of myeloid blasts in the bone marrow. According to the French-American-British
(FAB) cooperative group the diagnosis of AML requires at least 30% myeloid blasts in the bone
marrow (41, 42). The classification by WHO is based on morphology, histochemistry and
cyto genetics. The WHO system defines four major categories of AML, namely; i) AML with
recurrent genetic abnormalities, ii) AML with multilineage dysplasia, iii) therapy related AML
and iv) AML not otherwise categorized.

French-American-British (FAB) Classification: In the 1970s, a group of French, American,
and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7,
based on the type of cell from which the leukemia developed and how mature the cells are. This
was based largely on how the leukemia cells looked under the microscope after routine staining.
The French-American-British (FAB) classification divided AML into 8 subtypes designated as
M0 to M7 (41) listed in table 1 below.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO</td>
<td>Undifferentiated leukemia; Myeloperoxidase negative Myeloid markers positive</td>
</tr>
<tr>
<td>M1</td>
<td>Myeloblastic without differentiation; immature white blood cells</td>
</tr>
<tr>
<td>M2</td>
<td>Myeloblastic with differentiation; can be divided into those with t(8;21) AML1-ETO fusion</td>
</tr>
<tr>
<td>M3</td>
<td>Promyelocytic; APL; most cases have t(15;17)(q24;q21) PML-RARα or another translocation involving RARα</td>
</tr>
<tr>
<td>M4</td>
<td>Myelomonocytic; myelodysplastic syndrome</td>
</tr>
<tr>
<td>M4eo</td>
<td>Myelomonocytic with bone marrow eosinophilia; Characterized by inversion of chromosome 16 involving CBFb, which forms a heterodimer with AML1</td>
</tr>
<tr>
<td>M5</td>
<td>Monoblastic leukaemia; involves t(9;11) translocation</td>
</tr>
<tr>
<td>M5a</td>
<td>Monoblastic without differentiation</td>
</tr>
<tr>
<td>M5b</td>
<td>Monoblastic with differentiation</td>
</tr>
<tr>
<td>M6</td>
<td>Erythroleukemia (M6a), pure erythroid leukemia (M6b)</td>
</tr>
<tr>
<td>M7</td>
<td>Megakaryoblastic leukemia; associated with GATA1, t(1;22)(p13;q13) mutation. Increased risk in those associated with Down’s syndrome.</td>
</tr>
</tbody>
</table>
The molecular changes that occur in AML patients with no detectable cytogenetic abnormalities make up the largest subgroup and represent a variety of cytogenetically silent molecular genetic abnormalities. The number of known mutations associated with AML continue to grow at an unprecedented pace with over 300 different chromosomal translocations and other mutational events having already been described. Therefore, it is obvious that there are many more leukemic genotypes than phenotypes. Gilliland (2001) proposed a ‘two-hit’ model (figure 2) for leukemogenesis in an attempt to provide a unified molecular theme to explain how different mutations can generate essentially similar phenotypes.

Class I mutations
Providing proliferative and/or survival advantage
- BCR-ABL
- N-RAS or K-RAS
- c-Kit (exon 8)
- c-Kit (Asp 816)
- FLT3-ITD
- FLT3 (Asp 835)
- PTPN11
- NF1
- TEL-PDGFRβ

Class II mutations
Impairing differentiation and subsequent apoptosis
- CBFβ-MYH11
- AML1-ETO
- TEL-AML1
- PML-RARα
- NUP98-HOXA9
- PU.1
- C/EBPα
- AML1
- AML1-AMP19

AML 1-COPINE VIII

Drug; e.g. Flt3 inhibitor

AML

Drug; e.g. ATRA

Figure 2: The “two-hit” model of AML development. Adapted from Gilliland et.al. Blood, 2002 (43-45). Class I mutations include mutations which confer a growth advantage whereas class II mutations impair the hematopoietic differentiation. AML1- COPINE VIII is a unique mutation with a resulting fusion protein with both class I and class II activities (46).

The basis of the hypothesis is that AML is the consequence of a collaboration between at least two broad classes of mutation; class I mutations that confer a proliferative and/or survival
advantage to cells (BCR-ABL, N-RAS, KRAS, c-Kit (exon 8), c-Kit (Asp816), FLT3-ITD, FLT3 (Asp835), PTPN11, NF1 and TEL-PDGFR.) and class II mutations that primarily impair haematopoietic differentiation and cellular apoptosis (CBFβ–MYH11, AML1–ETO, TEL–AML1, PML–RARα, NUP98–HOXA9, PU.1, C/EBPα, AML1 and AML1–AMP19) (45). The model predicts that AML results from the combined effects of only two mutations, one from each class. However, a limitation of the model has been the lack of identifiable class I and class II mutations in the majority of AML cases. The mutations of receptor tyrosine kinases (RTKs) class III and RAS frequently provide the 'missing' proliferative signal in AML. Mutated tyrosine kinases such as FLT3 or kit, activated alleles of N-RAS or K-RAS and constitutive expression of transcription factors, such as NF-kB have been found in a significant percentage of AML patients. The mutations are associated with a significantly greater risk of relapse and reduced survival (44).

1.2.2.1 Prognostic factors

The median survival for AML patients receiving supportive therapy alone is only 3-4 months and very few patients survive for more than one year. The prognostic factors include the cytogenetic test (showing chromosome or gene changes), the patient's age and the white blood cell count. Other important factors include pre-existing blood disorders (such as a myelodysplastic syndrome) and a history of treatment with chemotherapy and/or radiation therapy for an earlier cancer. There are numerous chromosomal aberrations and other genetic defects detected in AML; recurrent cytogenetic abnormalities are used in prognosis and guidance for therapeutic decisions on hematopoietic stem cell transplantation or intensive chemotherapy alone.

Chromosome abnormalities

Chromosome changes give one clue to prognosis; however, not all patients have these abnormalities. Based on the cytogenetics, patients can be classified into three major subgroups with different prognosis. The group with favorable cytogenetics includes about 25% of the patients. Examples of favorable cytogenetics are t(8; 21)(q22; q22) and t(15; 17)(q22; q21) which creates the fusion protein AML1-ETO (47) and PML-RARα (48) fusion proteins respectively. Patients with latter fusion protein receive targeted therapy with all-trans retinoic
acid (ATRA) in addition to conventional therapy. The adverse cytogenetic group includes about 10% of AML patients with multiple abnormalities as deletions of either chromosome.

**Favorable abnormalities:**
- Translocation between chromosomes 8 and 21; t(8; 21)
- Inversion of chromosome 16
- Translocation between chromosomes 15 and 17; t(15; 17)

**Unfavorable abnormalities:**
- Complex changes - involves several chromosomes.

**Gene mutations**

Several molecular genetics aberrations also have a prognostic impact in AML. The most important genetic aberration is in-frame internal tandem duplications (ITDs) of the receptor tyrosine kinase FLT3 (44). FLT3 mutations are associated with an adverse prognosis, and are the strongest separate marker for disease relapse in AML. Another frequent genetic aberration in AML is mutations in the nucleophosmin gene (NPM1) (49). These mutations are present in 40 – 50% of AML patients with normal karyotype and represent a favorable prognostic factor for patients without FLT3 mutations. Several other molecular prognostic markers indicated are: Partial tandem duplications of the mixed lineage leukemia (MLL) gene associated with a short remission duration (50). High expression of the Brain and Acute Leukemia Cytoplasmic (BAALC) gene is an adverse prognostic factor (51). Mutations in CCAAT/enhancer binding protein alpha (CEBPA) give a favorable prognosis of disease outcome (52). Mutations in TP53 are associated with secondary leukemia and chemo resistance (53). Gene expression profiling by DNA microarrays is a new method that is increasingly used for prognostic evaluation and identification of novel subclasses of AML. Gene expression profiling signatures have been correlated to clinical outcome in several studies and will probably become a valuable tool for future molecular diagnostics.

**Other Factors**

Age is also one of the many factors since older patients (over 60) generally do not fare as well as younger patients. Some of this may be because they are more likely to have unfavorable
chromosome abnormalities. Older patients may also have other medical conditions that can make it harder to treat them with more intense chemotherapy regimens. Having a prior blood disorder such as a myelodysplastic syndrome or having AML that develops after treatment for another cancer tends to lead to a worse prognosis because these types of AML are often harder to treat.

1.2.2.2 Treatment of AML

AML is not a single disease but a number of related diseases each distinguished by unique cytogenetic markers which in turn help determine the most appropriate treatment. These cytogenetic abnormalities, as well as other mutations, give rise to abnormal signal transduction and these abnormal pathways represent ideal targets for the development of new therapeutics. The chemo drugs used most often to treat AML are cytarabine (cytosine arabinoside or ara- C) and the anthracycline drugs (such as daunorubicin/daunomycin, idarubicin, and mitoxantrone). These chemotherapeutic drugs have various side effects which include; hair loss, mouth sores, loss of appetite, nausea, vomiting and it also affects bone marrow, which often causes low blood cell counts in AML patients (54).

Acute Promyelocytic Leukemia with -t(15;17) translocation leading to formation of a fusion protein PML-RARα is the subtype of acute leukemia where the greatest progress has been made over the past decade (48). It is the most curable subtype of AML and the most important development leading to the dramatic improvement in survival has been the introduction of all-trans retinoic acid (ATRA) (55). ATRA is a form of vitamin A that is often part of the initial treatment of APL (56). It is often given along with chemo - an anthracycline drug with or without cytarabine. For patients who can't take an anthracycline (often because of heart problems), ATRA can also be given with arsenic trioxide for the initial treatment of APL, in which no regular chemo drugs are given (57). ATRA can have side effects which include headache, fever, dry skin and mouth, skin rash, swollen feet, sores in the mouth or throat, itching and irritated eyes. It can also cause blood lipid levels (like those of cholesterol and triglycerides) to go up. Often blood liver tests become abnormal.

While the incorporation of ATRA has led to these remarkable results, differentiation therapy with ATRA is associated with unique toxicities not previously observed with conventional cytotoxic therapy. AML is marked by the block in differentiation and it often
shows some morphological signs of differentiation induced by exposure to various soluble mediators like trans-retinoic acid (ATRA) and several cytokines (58). Several agents can induce leukemic cell differentiation in other AML subtypes, although these effects may differ between patients. Differentiation may then be associated with induction of apoptosis and differentiation-inducing therapy may therefore become useful in combination with intensive chemotherapy to increase the susceptibility of AML blasts to drug-induced apoptosis.

Bone marrow transplant is also effective in the treatment of AML (59). New therapeutics used in the treatment of AML includes antibody-based or cell-based immunotherapy, drug conjugates, radioimmunoconjugates, T cell adaptive immunotherapy and AML vaccines (60-63). Future studies in AML should therefore focus on: A) the identification of new agents with more predictable effects on differentiation and apoptosis; B) the use of clinical and laboratory parameters to define new subsets of AML patients in which differentiation/apoptosis induction has a predictable and beneficial effect, and C) further characterization of how AML blast sensitivity to drug-induced apoptosis is modulated by differentiation induction. In addition, differentiation therapy is also considered for other AML patients as a therapeutic approach with low treatment-related morbidity and mortality. Therefore, screening of compounds using in-vitro assays having potential to induce apoptosis and differentiation in myeloid blasts may have substantial impact in the development of better therapeutics for leukemia.

1.2.3 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of haematopoietic stem cells (64). This disease evolves in 3 distinct clinical stages: a chronic phase (CP) lasting three to six years is followed by transformation to an accelerated phase (AP) and then a terminal blast phase of short duration (65, 66). CML is one of the best understood diseases from the aspect of its cytogenetic abnormalities and the molecular mechanisms involved. CML is consistently associated with an acquired genetic abnormality, the Philadelphia chromosome (Ph1), a shortened chromosome 22 resulting from a reciprocal translocation of the long arms of chromosomes 9 and 22 (67). This translocation generates the BCR/ABL fusion gene, which is translated in p210BCR/ABL oncoprotein (figure 3) (68). Expression of p210BCR/ABL is necessary and sufficient for malignant transformation, as demonstrated with in vitro assays and
leukemogenesis in mice (69-71). BCR-ABL oncoprotein, a constitutively active tyrosine kinase recruits and activates several pathways transducing intracellular signals which ultimately lead to abnormal cellular adhesion, enhanced proliferation and inhibition of apoptosis. The BCR/ABL fusion protein helps in the proliferation and survival of myeloid progenitor cells by activating RAS and PI-3K/AKT pathway (72-74). Src family kinase interacts with BCR/ABL, gets activated which in turn phosphorylates STAT5B and thereby increases the expression of anti-apoptotic Bcl-XL protein (75, 76).

**Figure 3**: Philadelphia chromosome (Ph) caused by reciprocal translocation between chromosomes 9 and 22 is generally believed to be the sole cause of CML (adapted from Druker, B. J. Adv. Cancer Res. 2004, 91, 1-29).

Transition from chronic to blast crisis is unavoidable outcome of CML except in a cohort of patients receiving allogenic bone marrow transplants early in chronic phase. Two other BCR/ABL proteins, p190 and p230, generated by variant fusion genes, are only occasionally detected in CML (77-79).

The treatment of CML has long been in the frontier of cancer therapy. Imatinib mesylate (STI571, imatinib, Glivec®, or Gleevec®, Novartis, Basel, Switzerland) is the treatment of choice for chronic-phase CML and due to its remarkable therapeutic effects; blast crisis
transition is postponed for several years in most patients with CML (80-82). However, Imatinib therapy alone cannot be the only solution to the treatment of CML due to growing evidences of drug resistance developed in CML patients. Therefore, screening of compounds which may kill Philadelphia chromosome harboring cells or may induce differentiation in arrested cells can be boon for such patients.

1.2.3.1 Pathology of CML

Philadelphia chromosome discovered by Peter Nowell in 1960 provided first evidence for genetic link of cancer (67). The leukemogenic potential of p210 \(^{BCR-ABL}\) resides in the fact that the normally regulated tyrosine kinase activity of the ABL protein is constitutively activated in the fusion oncoprotein (83). ABL proteins are non-receptor tyrosine kinases that have important roles in signal transduction and the regulation of cell growth. There are two isoforms of ABL, isoform 1a and isoform 1b. Isoform 1b, which is expressed at higher levels in early haematopoietic progenitor cells, is myristoylated on its second glycine residue at the N-terminal (84). Loss of myristoylation in ABL dramatically enhances its tyrosine kinase activity (85). Downstream to the myristoylation site, at the N-terminal segment of ABL, there are three SRC homology domains (SH3, SH2 and SH1). SH2 and SH3 regulate the tyrosine kinase function of ABL and SH1 harbors the tyrosine kinase activity of ABL. SH3 has a negative regulatory effect on the tyrosine kinase function. Deletion of SH3 or mutation in SH3 facilitates tyrosine kinase activity of ABL (84-86). Defects in the functional integrity of SH2 decrease phosphotyrosine binding and reduces the transforming capacities of ABL (87). The C-terminal part of ABL contains a DNA-binding domain, nuclear localization signals, and a binding site for actin (88). The disruption of ABL protein by genetic fusion is responsible for the up-regulated tyrosine kinase activity. The uncontrolled tyrosine kinase activity of BCR-ABL is also caused by the juxtaposition of alien BCR sequences. The N-terminal coiled-coil motif of BCR promotes dimerization and increases BCR-ABL tyrosine kinase activity and enables binding of F-actin to ABL (89). The serine-threonine kinase domain of BCR activates signaling pathways mediated by BCR-ABL tyrosine kinase (90). BCR which also contains SH2 binding sites, fusion to ABL adds a large amino acid sequence to the SH2 segment of ABL. BCR interferes with the adjacent SH3 and SH2 kinase regulatory domain, which in turn causes ABL to become constitutively active as
a tyrosine phosphokinase (91). Ectopic expression of BCR/ABL in growth factor-dependent cell lines activates numerous signal transduction pathways responsible for growth factor independence and reduced susceptibility to apoptosis of these cells (92).

1.2.3.2 Treatment of CML

Cytoreductive chemotherapies, INF-α and allogeneic stem cell transplantation were the standard treatment options for CML patients in chronic phase before the imatinib era. IFN-α is a member of glycoprotein family which has antiviral and antiproliferative properties. IFN-α was first shown to be an active agent in CML in the early 1980s and it became the non-transplant treatment of choice for chronic phase CML patients (93). IFN-α has been shown to increase survival while haematologic responses are seen in the majority (80%) of patients; cytogenetic responses are seen in only 30-50% of patients with complete cytogenetic responses in only 10-20% of IFN-treated patients (94-96). Unfortunately many patients tolerate IFN-α poorly, necessitating dose reduction or discontinuation of treatment. Currently, the only curative approach for CML is allogeneic stem cell transplantation. The outcome of this procedure depends on a series of risk factors, the most important of which are the patient’s age and the phase of the disease (97). For young (age <40 years) patients undergoing transplants within 1 years from diagnosis, long-term survival rates are reported to be 70-80%. With advances in molecular HLA-typing, improvements in infection control and graft versus host disease prophylaxis, outcomes for related and unrelated donor transplants appear similar. Unfortunately, up to one third of CML patients are over the age of 60 for whom the allogeneic stem cell transplantation usually is not feasible due to high risk of treatment-related mortality (98). Therefore, for many patients with CML, stem cell transplantation is not an option.

1.2.3.3 Imatinib mesylate

Imatinib (STI571, Gleevec) is the first successful, rationally developed, receptor-targeted agent for chronic myelogenous leukemia (CML). Imatinib was initially developed by scientists at Ciba-Geigy (currently Novartis, Basel, Switzerland), as a specific platelet-derived growth factor receptor (PDGFR) inhibitor (82). As the BCR-ABL tyrosine kinase plays a key role in CML pathogenesis, attempts to target the BCR-ABL tyrosine kinase evolved as new therapeutic
strategies. It was also found to be a potent ABL tyrosine kinase inhibitor. Further optimization for v-ABL tyrosine kinase inhibition led to generation of imatinib mesylate which selectively inhibits ABL tyrosine kinase including BCR-ABL (figure 4) (81, 99).

![Diagram of BCR-ABL signaling](image)

**Figure 4:** Imatinib a specific inhibitor of small family of tyrosine kinases, including BCR-ABL blocks the ATP binding site and prevents substrate phosphorylation, thereby interrupting BCR-ABL signal transduction pathways that lead to leukemic transformation (adapted from Schindler, T. et al. Science, 2000).

Further studies revealed that a limited number of other tyrosine kinases are also targeted by imatinib, including PDGFR (82), c-KIT (100) and ARG (101). Preclinical studies showed that imatinib selectively inhibits the proliferation of cell lines holding \( p210^{\text{BCR-ABL}} \) and the clonal growth of myeloid cells from CML patients (102). It was also shown in mice models that imatinib had *in vivo* activity against *BCR-ABL* positive cells and that continuous exposure to imatinib was necessary to eradicate the tumors, suggesting this would be important for an optimal antileukemic effect (103). Though imatinib mesylate is widely used as a treatment of
choice for the CML, there are some side effects which act as a limiting factor in its therapeutic use.

(i) Molecular persistence:

Chronic phase patients treated with imatinib mesylate remain positive when tested by qRT-PCR for BCR-ABL transcripts though they have been expected to achieve complete cytogenetic remission i.e. they have a persisting minimal residual disease (104). Even those who have undetectable BCR-ABL transcripts may still harbor as many as $1 \times 10^7$ leukemic cells in their bodies and there is a high likelihood for relapse if the drug is stopped (105-107). Bone marrow studies have shown that the residual Ph-positive cells are part of the leukemic stem cell compartment (108, 109). Studies performed in vitro suggest that many primitive Philadelphia-positive progenitor or stem cells are relatively insensitive to imatinib (110).

(ii) Resistance:

Primary and acquired resistance can be seen in all stages of CML patients treated with imatinib mesylate. Resistance to imatinib is multifaceted. Generally, there are two types of resistance, primary and acquired. Primary resistance may be defined at the haematologic, cytogenetic or molecular levels. Acquired resistance can be defined as: (i) progression into blast crisis; (ii) loss of a sustained CHR or cytogenetic response; and (iii) a 5- to 10-fold rise in BCR-ABL transcript number (111). However, the mechanism of primary resistance is still mainly unsolved. In general, there are two possible categories of the molecular mechanisms of imatinib resistance, i.e. BCR-ABL independent and BCR-ABL dependent (112). In the first category, secondary oncogenic changes can occur in the leukemic cells and render the cell proliferation independent of BCR-ABL. In this scenario, BCR-ABL is no longer a relevant target and even the most ideal BCR-ABL inhibitor would be ineffective in this setting. However, BCR-ABL-independent mechanisms are rare events. In the second, BCR-ABL-dependent category, some change is predicted in either the patient (host-mediated) or the leukemic clone (cell-intrinsic) that prevents the drug from effectively shutting down the target BCR-ABL protein. Host-mediated resistance can occur through enzymatic modification of imatinib by a P450 enzyme in the liver or by production of a protein that neutralizes drug activity, such as alpha-1 acid glycoprotein (113-
115). Cell-intrinsic resistance could occur by modification of the target BCR-ABL tyrosine kinase through gene amplification or BCR-ABL kinase domain mutations or by a reduction of intracellular drug concentration through over expression of multidrug resistance genes (116). Among these mechanisms, the BCR-ABL kinase domain mutations are the most studied. These different lines of evidence suggest that imatinib, although being highly active against the differentiated mass of CML cells, probably fails to eradicate leukemic stem cells (117-119).

In CML, the leukemic stem cells can be considered quiescent, spending most of their time in G0. Under certain circumstances, leukemic stem cells can enter cell cycle and give rise to progenitors, which produce differentiated leukemic cells (120, 121). Expansion of Ph-positive progenitors is inhibited by imatinib and life-long imatinib therapy is likely required to continuously suppress the remaining leukemic cells in CML patients, even in the best responders. Development of treatment targeting these quiescent stem cells, e.g. immunotherapy, is a challenge in CML.

1.3 Ormeloxifene

Ormeloxifene (C30H35O3N.HCl; trans-1-[2-\{4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2H-1-benzopyran-4-yl)-phenoxy\}-ethyl]-pyrrolidine hydrochloride; also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control (122). It was synthesized at CSIR-Central Drug Research Institute, Lucknow (123). This agent is a unique need-oriented contraceptive agent which is included in the National Family Welfare Programme of India. Since 1991, ormeloxifene has been used as a need-oriented contraceptive and is being given for treating dysfunctional bleeding of the uterus. This agent's contraceptive activity is well established in rodents and primates (124). Ormeloxifene inhibits implantation via inhibition of endometrial receptivity to blastocyst signals by antagonism of the action of estrogen, without altering the concentration or secretion pattern of estrogen and progesterone, hypothalamo-pituitary-ovarian axis, follicle maturation, ovulation, mating behavior, gamete transport or fertilization, and pre-implantation development of embryos (125-127). Clinically, ormeloxifene has been reported to provide good pregnancy protection in women in postcoital as well as weekly regimens and is marketed in India as a contraceptive pill.
Ormeloxifene is efficient in the management of hormone related clinical disorders (129). It is effective in the treatment of polycystic ovarian syndrome by induction of ovulation (130). Due to its potent anti-estrogenic and weak estrogenic activities (127, 131, 132), it is also effective against advanced breast cancer (133) and may be therapeutically effective for other clinical conditions such as dermatitis, osteoporosis, restenosis, endometriosis and uterine fibroids. It prevents bone loss by directly inhibiting the bone reabsorbing activity of osteoclast. Ormeloxifene as a racemate has been found to be a potent cholesterol-lowering pharmaceutical resulting in a significant decrease in serum concentrations. Ormeloxifene has also been reported to be efficacious in the management of dysfunctional uterine bleeding or menorrhagia. In nearly 78% percent of the patients the menorrhagia was cured without any side effect (134). Thus, it acts as an effective and safe therapeutic option for the medical management of menorrhagia. Ormeloxifene suppresses the receptors in the reproductive organs like the ovaries, uterus and breasts while it stimulates the estrogen receptors of other organs like the bones (129, 135, 136). Substantial evidence has been amassed to support the premise that ormeloxifene has potential anticancer activity (128, 133). In a published study, Mishra et. al, evaluated the role of ormeloxifene in breast cancer patients. Apparently, treatment with ORM was evaluated in 4 male and 75 female patients with advanced breast cancer. The overall response rate, including both male and female cases, was 40.5%. Among the female patients, the overall response rate was
38.7%. One of the 4 male patients showed a complete response and 2 showed partial responses. The responses were more marked for bone, pulmonary, soft tissue, skin and lymph-node metastases than for liver metastases (133). In addition, ORM can reduce the mutagenic effects of known genotoxic compounds dimethylbenz[a]anthracene (DMBA), cyclophosphamide (CP), mitomycin C (MMC) and ethyl methanesulfonate (EMS) in *Salmonella*. These protective activities of ORM against the known positive mutagens in the *Salmonella* may be due to induction of apoptosis by ORM, which leads to the elimination of cells damaged by these mutagens from the cell population (128).

Ormeloxifene possesses excellent therapeutic index and has been well-tolerated, without any haematological, biochemical or histopathological evidence of toxicity during phase III trial in pregnant females. Ormeloxifene has no side effects after prolonged use. It is devoid of side effects such as nausea, headache, vomiting, dizziness, breakthrough bleeding, depression, mood change, nostalgia, and poor libido; androgenic ill effects such as acne and hirsutism, metabolic complications such as weight gain, hypertension, coagulation disorders, hyperglycemia, and abnormal lipid profile or pigmentation or ophthalmological or audiological effects.