AMERICAN UNIVERSITY OF BEIRUT

ARSENIC TRIOXIDE AND INTERFERON ALPHA CIRCUMVENT TYROSINE KINASE INHIBITORS RESISTANCE IN CHRONIC MYELOID LEUKEMIA MODELS

by
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AN ABSTRACT OF THE THESIS OF

Nagham Rasbieh for Master of Physiology
Major: Physiology

Title: Arsenic trioxide and interferon alpha circumvent tyrosine kinase inhibitors resistance in chronic myeloid leukemia models.

Background and Aims: Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by t (9; 22) translocation that generates bcr-abl fusion gene coding for BCR-ABL oncoprotein with abnormal constitutive tyrosine kinase activity. Tyrosine kinase inhibitors (TKI) have been successfully established for the treatment of CML. Despite high rates of clinical response, CML patients can develop resistance against TKI mainly due to kinase domain mutations. Of special interest is T315I mutation, which accounts for 15–20% of mutations affecting ABL kinase domain. T315I confers resistance to almost all TKI. Ponatinib, the only TKI effective against T315I single but not T315I-inclusive compound mutations, was suspended due to its cardiac side effects and is currently limited to specific cases.
Recently, we demonstrated that arsenic trioxide (ATO) and interferon alpha (IFNα) inhibited proliferation, induced apoptosis, prolonged survival and affected leukemia initiating cells activity in wild-type bcr-abl CML models. Here, we investigate the effect of ATO and IFNα on the proliferation, cell cycle arrest and induction of apoptosis in imatinib-resistant CML cell lines and its anti-tumor activity in CML mouse model harboring the T315I mutation.

Methods: Imatinib-resistant K562-R and Ar230-R CML cells were treated with different concentrations of ATO and IFNα. The effect of the treatment on cell proliferation was performed using MTT assay. Synergy analysis was calculated using the compusyn software. The effect of the treatment on cell cycle arrest and apoptosis was performed using PI assay, TUNEL assay and Rhodamine-123 assay, respectively. Using a retroviral bcr-abl T315I transduction murine CML model, we studied the effect of ATO/IFNα on the survival of leukemic mice harboring this famous mutation.

Results: Our preliminary results demonstrated that ATO and IFNα synergized to inhibit the proliferation of imatinib-resistant CML cells. In addition, ATO/IFNα combination induces G2/M cell cycle arrest and apoptosis that was accompanied by loss of mitochondrial membrane potential. Importantly, this combination significantly prolongs the survival of CML mice carrying the T315I mutation.

Conclusion: Our preliminary data provide clear evidence demonstrating a potential preclinical efficacy of ATO/IFNα in TKI-resistant CML models, specifically in CML mouse models with the T315I mutation, resistant to all available primary and secondary TKI.

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ABBREVIATIONS

5-FU	5-fluorouracil
ABC	ATP binding cassette
Abl	Abelson kinase
ALL	Acute lymphoblastic leukemia
AP	Accelerated phase
APL	Acute promyelocytic Leukemia
ATL	Adult T-cell Leukemia
ATO	Arsenic Trioxide
ATP	Adenosine triphosphate
BC	Blast crisis
Bcl-2	B-cell lymphoma-2
bcr-abl	Breakpoint cluster region-abelson gene
BCR-ABL KD	Breakpoint cluster region-abelson gene Kinase Domain
BMC	Bone marrow cell
CBC	Cell Blood Count
CCgR	Complete cytogenetic response
CHR	Complete hematologic response
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMR	Complete molecular response
CP	Chronic phase
CTL	Cytotoxic T lymphocytes
dHSC	Dormant haemopoietic stem Cells
DNA	Deoxyribonucleic acid
FACS	fluorescence-activated cell sorting
FADD	Fas associated death domain
FAS	First apoptosis signal
FasL	Fas ligand
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GFP	Green Fluorescent Protein
HSC	Haemopoietic stem cells
IL-3	Interleukin 3
IL-6	Interleukin 6
IRES	Internal Ribosomal Entry Site

JAK	Janus kinase
LSC	Leukemic stem cells
MCgR	Major cytogenetic response
mCgR	Minor cytogenetic response
MMR	Major molecular response
PBS	Phosphate Buffered Saline
PCgR	Partial cytogenetic response
PCR	Polymerase chain reaction
PHR	Partial hematologic response
PML	Promyelocytic leukemia protein
PML-RARA	Promyelocytic leukemia protein-Retinoic acid receptor alpha
PI	Propidium Iodide
ROS	Reactive oxygen species
STAT	Activators of transcription
SCF	Stell Cell Factor
TKI	Tyrosine kinase inhibitors
TRAIL	TNF-related apoptosis-inducing ligand

Chapter I

Introduction

A. Haemopoiesis and Haemopoietic Stem Cells

Haemopoiesis is the process of blood cell formation [1]. It is also viewed as the process of development, self-renewal, and differentiation of haemopoietic stem cell (HSC) [2]. HSCs account for no more than 0.05% of haemopoietic cells and are responsible for the constant renewal of all blood cell lineages [3]. Most HSCs are inactive in terms of cell cycle activity [4]. Indeed, long-term labeling studies have demonstrated that the most potent HSCs in the adult healthy mouse are in a state of dormancy [5]. These dormant HSCs (dHSCs), which show the highest self-renewal potential of all HSCs, are found as individual cells in niches within the cavities of the trabecular bone where they divide only about five times per lifetime, and are almost permanently in the G0 phase of the cell cycle [6].

Why does the body keep such a reservoir of dormant HSCs in the adult bone marrow? It turns out that loss of haemopoietic cells by bleeding, toxic insults or chemotherapy induces a feedback loop leading to the awakening of dHSCs to restore the HC loss [7]. In addition, most mature blood cells have limited lifespan in the peripheral circulation; therefore to maintain the haemopoietic system in steady state, new blood cells are generated in the bone marrow by the proliferation, lineage commitment, and differentiation of HSC. It is reported that in a healthy adult, about 1.5 x 10^6 blood cells are continuously generated every second [8]. Thus dHSCs most likely serve as a reservoir of highly potent but well protected HSCs essential for rapid and

efficient reconstitution of the blood system in response to severe and life threatening bone marrow stress [5, 7].

HSC pool is maintained and regulated by their asymmetric cell division which enables them to simultaneously self-renew and generate progenitor cells [6]. Yet many stem cells can also divide symmetrically to generate two identical daughter stem cells or two progenitor cells, especially when they are expanding in number during development or after injury [9] (Figure 1). In addition, HSCs can undergo apoptosis due to DNA double strand breaks during aging. In other words, HSC can remain dormant, enter cell cycle to undergo symmetric or asymmetric division, or undergo apoptotic death. The fate of haemopoietic stem cells is thought to be regulated by factors in the bone marrow microenvironment, i.e. stem cell niche [10].

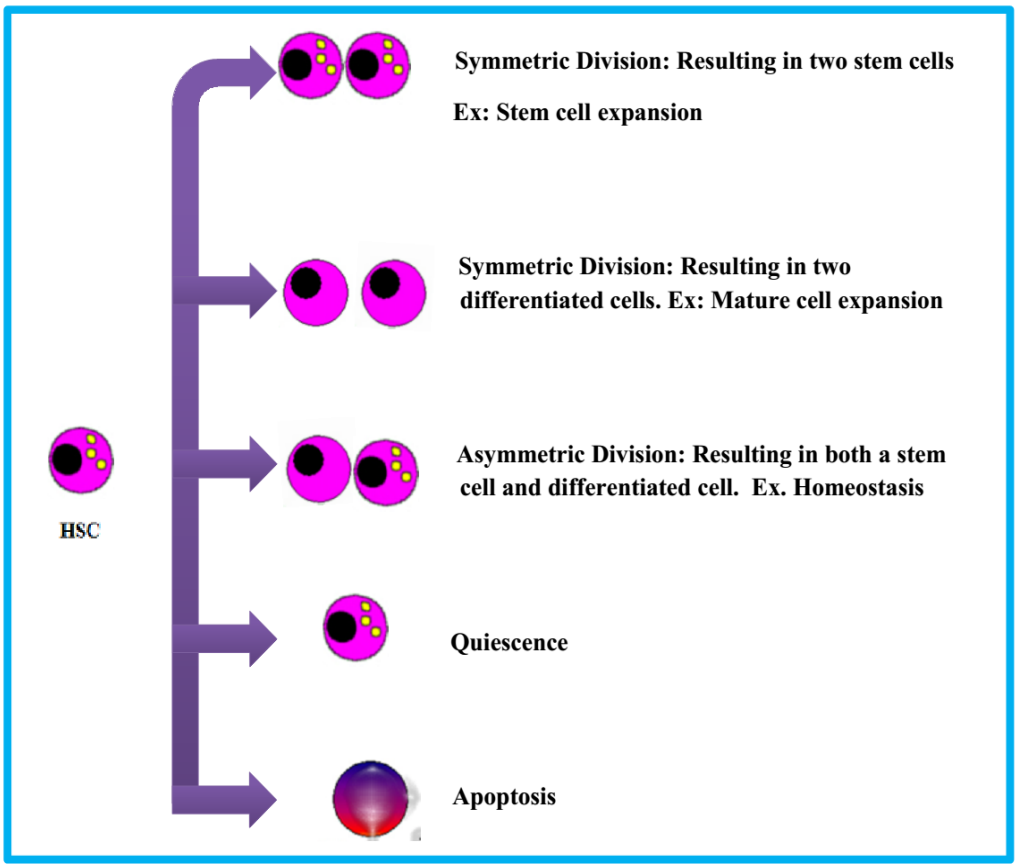


Figure 1: Possible HSC fates.

HSCs are characterized by increased cell cycle quiescence compared to other cells. They can remain in quiescence, enter cell cycle to undergo symmetric or asymmetric division, or undergo apoptotic death.

Murine HSCs show a Lin^- $Sca1^+$ $cKit^+$ $CD34^-$ $CD150^{hi}CD48^-$ phenotype [5,7, 11-14]. Human HSCs are less well defined but represent >10% of the $Lin^-CD34^+CD38^-$ $CD90^+CD45RA^-$ population within cord blood [15, 16]. In the haemopoietic system, stem cells can be divided into three populations based on their ability to self-renew: long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors without detectable self-renewal potential [17, 18]. As HSCs mature from the long-term self-renewing pool to multipotent progenitors, they progressively lose their potential to self-renew but become more mitotically active. The three populations also

differ in their ability to reconstitute the haemopoietic system of lethally irradiated mice. The highly self-renewing long-term HSCs are able to restore haemopoiesis and give rise to all types of blood cells throughout the animal's entire life span, whereas the short-term HSCs and multipotent progenitors restore hemopoiesis for a limited period of time [19].

1. Leukemic Stem Cells

Leukemic stem cells (LSCs) are a small and unique population of cells that causes Leukemia. Since the early 1970s, the notion of tumorigenic LSCs has emerged based on several studies showing that only a small subset of leukemic cells was capable of extensive proliferation in vitro and in vivo. Park and coworkers showed that only 1 in 10,000 to 1 in 100 leukemic cells were able to form colonies in vitro in colony-forming assays [20].

LSCs derive either directly from HSCs that have acquired multiple mutations, during their normal developmental progression, or from committed progenitors that acquired self-renewal ability due to additional mutations (Figure 2) [21, 22]. LSCs are given this name not necessarily because they often arise from normal stem cells, but because they also share the same characteristics used to define normal stem cells. These cells have the ability to self-renew and are multipotent and highly proliferative. The general mechanisms underlying leukemic transformation are increased cell survival, increased proliferation capacity, increased self-renewal capacity, genomic instability, and prevention of differentiation [22]. From a therapeutic perspective, LCSs were shown to be resistant to most currently available leukemia treatments.

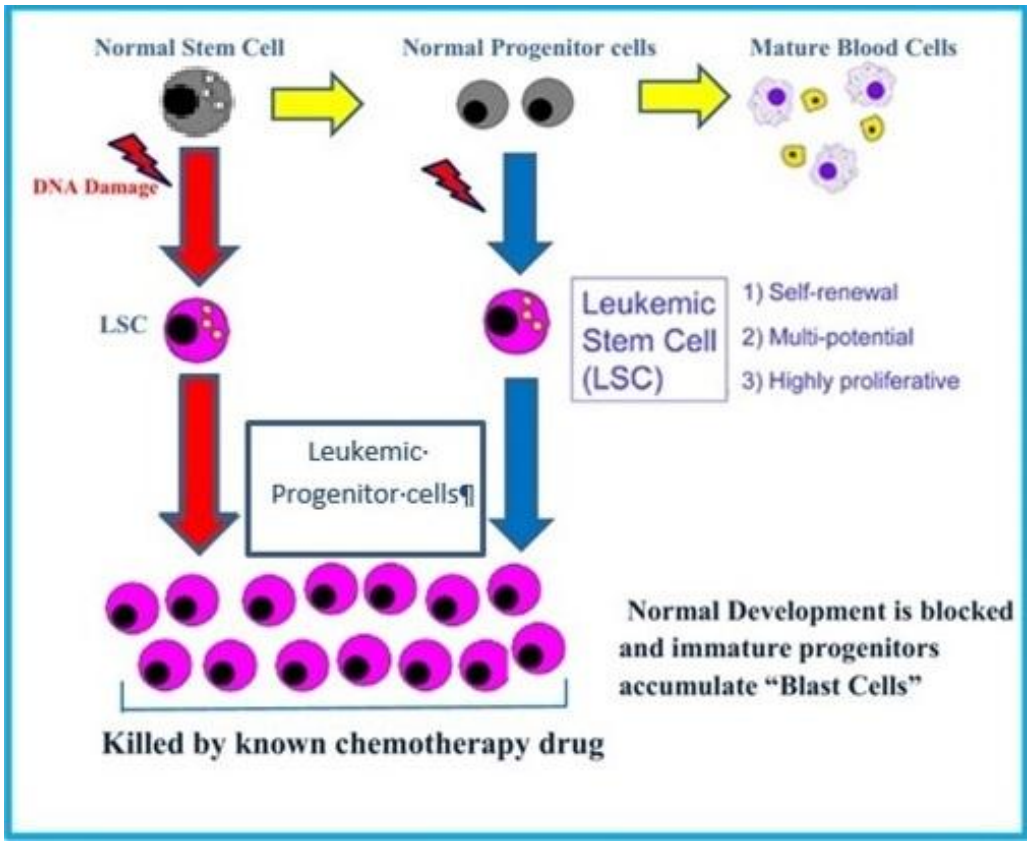


Figure 2: Origin of LSC.

LSCs can either derive from HSCs, which have become leukemic as the result of accumulated mutations, or more restricted progenitors, which have reacquired the stem cell capability of self-renewal.

B. Chronic Myeloid Leukemia

Chronic myeloid leukemia, known as CML, is a clonal hematoproliferative disorder [23, 24]. It is considered a relatively rare haemopoietic stem cell disorder with an annual incidence of 1-2 cases per 100,000 individuals [25], and occurs mostly in adults with rate of occurrence increases with age [26]. CML was first recognized, in 1845, by John Hughes Bennett [27] and David Craigie [28] who described it as a blood disease characterized by excessive and unregulated proliferation of white blood cell. In 1960, Nowell and Hungerford discovered the presence of a small abnormal chromosome, in the abnormal leukemic cells of CML patients, that was named Philadelphia (Ph) chromosome after the hometown where it was discovered [29]. In 1973, Rowley linked the underlying cause of CML to the reciprocal translocation between two chromosomes, 9 and 22, cytogenetically resulting in this abnormal Philadelphia chromosome [30]. Later, it was shown that a large part of the abelson (abl) gene on chromosome 9 is translocated to the breakpoint cluster region (bcr) gene on chromosome 22, giving rise to the chimeric bcr-abl oncogene (Figure 3)[31, 32].

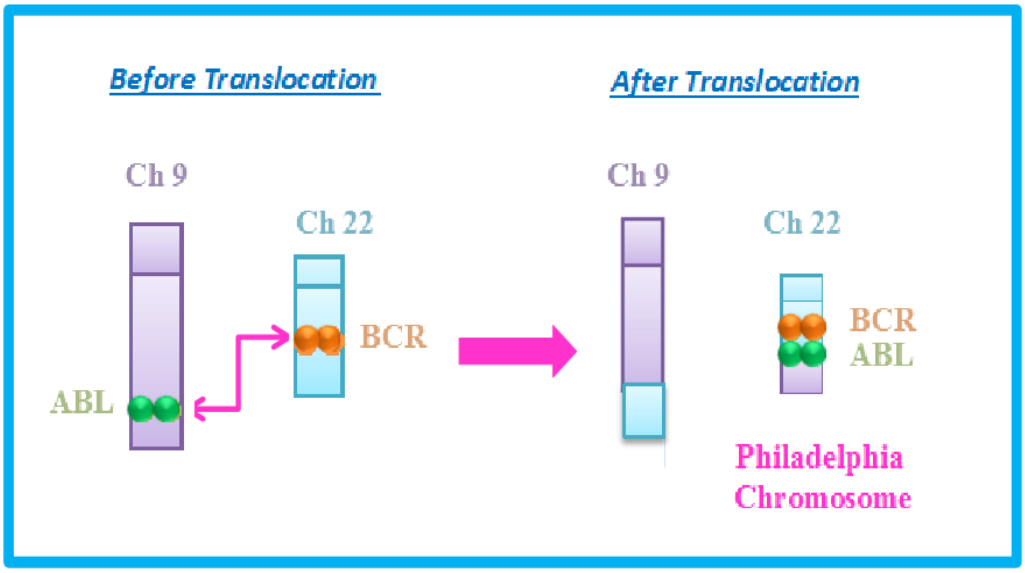


Figure 3: Development of Philadelphia chromosome.

In CML, the leukemic cells share a chromosomal abnormality not found in non-leukemic white blood cells, or any other cells of the patient's body. This genetic abnormality is due to a reciprocal translocation between chromosome 9 and chromosome 22 resulting in a Philadelphia chromosome that harbors the bcr-abl oncogene.

The bcr-abl oncogene exists in three forms: P190, P210 and P230 that arise from distinct breakpoints in the bcr gene. This mediates the production of three different BCR-ABL fusion proteins localized in the cytoplasm [30, 33], of molecular mass 190, 210, and 230 kD, respectively. The P210 form of bcr-abl oncogene is found in hemopoietic cells of patients with CML and acute lymphoid leukemia (ALL), however, the P190 form of bcr-abl oncogene is commonly found in ALL patients [5] but is rarely if ever observed in CML. BCR-ABL oncoprotein exhibits constitutively activated tyrosine kinase activity triggering enhanced proliferation, resistance to apoptosis, reduced growth-factor dependence, and perturbed interaction with extracellular matrix and stroma [34].

CML is a multi-stage disease, comprising early chronic phase (CP), secondary accelerated phase (AP), and advanced blast crisis (BC) [35]. Most CML patients are diagnosed in the chronic phase. Common signs and symptoms of CML in CP, when present, include anemia, splenomegaly [36], bleeding, fatigue, leukocytosis, weight loss, pain and thrombocytosis [37]. CP is characterized by an enhanced uncontrolled proliferation of myeloid lineage cells, causing over-production of mature granulocytes in the blood and bone marrow [37]. It is diagnosed upon examining a blood/ bone marrow sample, and most patients go to disease remission after treatment. However, if not efficiently treated, the disease would progress to an accelerated phase, then to a fatal blast crisis in about 3-5 years after onset [1]. Blast crisis phase is associated with a rapid and huge increase of blast cells in the blood, the growth of immature cells, and a less favorable response to treatment [38]. The mechanism of evolution of CP to blast crisis may involve genetic and epigenetic alterations including duplication of Ph chromosome, trisomy 8, mutations and deletions of tumor-suppressor genes such as p53 or p16, and deregulated expression of several transcriptional regulator including Fos and PRAME [38, 39]. Interestingly, recent publications suggest that during chronic phase CML, leukemic stem cells are the BCR-ABL positive haemopoietic stem cells. However, during blast crisis CML, in addition to the CML stem cells role in the propagation of the disease, the leukemic granulocyte macrophage progenitor (GMP) acquires stem cells properties such as self-renewal, increased proliferation and arresting differentiation leading to accumulation of immature blast cells, through acquiring additional mutations at the level of the β-catenin pathway [16] (Figure 4).

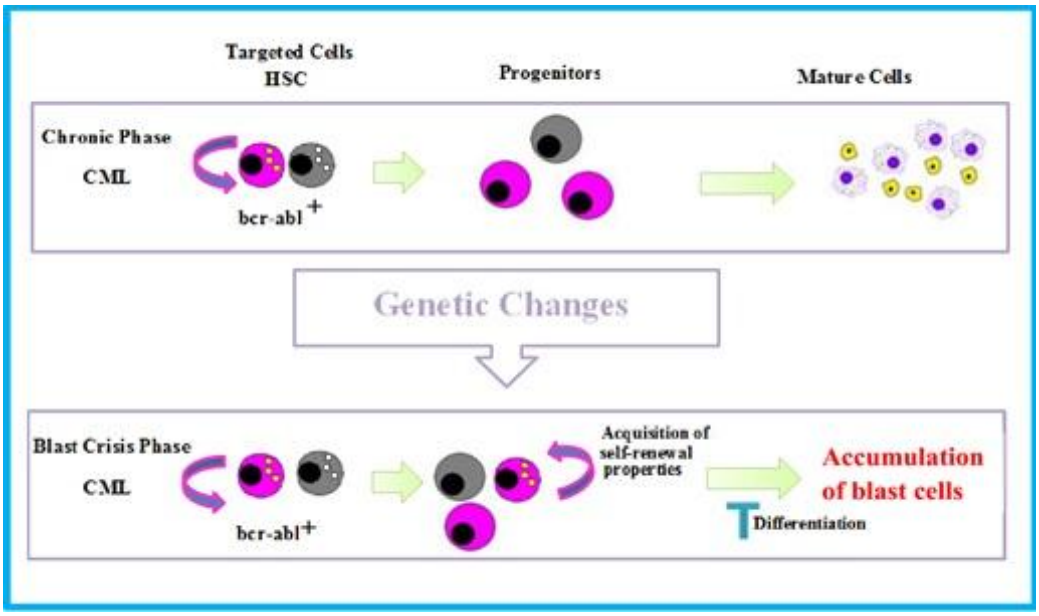


Figure 4: Chronic and Blast crisis CML.

The CML stem cell of CP gives rise to a progenitor population slightly more proliferative than the normal HSC. In advanced blast crisis CML, in addition to the role of the quiescent CML stem in propagation of the disease, the leukemic granulocyte macrophage progenitor acquires stem cell features leading to an expansive population of immature blasts.

Patients can be diagnosed with CML at hematological, cytogenetic and molecular levels. At the hematological level, a test is done on a blood sample taken from patient's peripheral blood, and called a complete blood count (CBC). In CML patients, a CBC test often shows high level of white blood cells, and abnormal levels of red blood cells and platelets. At the cytogenetic level, karyotyping of metaphase chromosomes is done to analyze the number, size and arrangement of chromosomes in the patient's blood cells. This technique is used to confirm the presence of Ph chromosome positive cells in CML patient's samples taken from peripheral blood or bone marrow. At the molecular level, CML patients usually undergo additional tests to confirm the presence of bcr-abl fusion gene. These tests include Fluorescence in situ

hybridization (FISH), and Polymerase chain reaction (PCR). FISH technique uses fluorescent probes to identify the presence of bcr-abl gene. On the other hand, PCR is another technique important to detect a certain DNA fragment through generating thousands of copies of this fragment, thus facilitating its discovery. These tests are not only necessary to confirm the diagnosis of CML, but also to monitor the treatment's effectiveness on CML patients and thus disease progression. The different criteria used to monitor treatment responses in CML patients are shown in Table 1.

Response	Criteria
Complete hematologic response (CHR)	**Complete normalization of peripheral blood count:** **● Leukocyte count less than 10 x 10^9 cells/L** **● Platelet count less than 450 x 10^9 cells/L** **● No immature cells in the peripheral blood** **● No signs or symptoms of disease including splenomegaly**
Partial hematologic response (PHR)	**● Persistence of immature cells, or** **● Platelet count is less than 50% of the pre-treatment count but more than 450 x 10^9 cells/L, or** **● Persistent splenomegaly but >50% of the pretreatment extent**
Complete cytogenetic response (CCgR)	**● 0% Ph^+ cells detectable in the bone marrow**
Partial cytogenetic response (PCgR)	**● 1-34% Ph^+ cells detectable in the bone marrow**
Major cytogenetic response (MCgR)	**● Less than 35% Ph^+ cells detectable in the bone marrow (includes CCgR and PCgR)**
Minor cytogenetic response (mCgR)	**● Between 35 and 90% Ph^+ cells detectable in the bone marrow**
Complete molecular response (CMR)	**● Undetectable bcr-abl transcript by qRT-PCR**
Major molecular response (MMR)	**● Equal or Less than 0.1% bcr-abl transcript by qRT-PCR**

Table 1: Criteria of treatment responses in CML.

1. CML in relation with BCR-ABL

BCR-ABL oncoprotein is found in 95% of CML patients, in 5% of children and 15-30% of adults with acute lymphoid leukemia (ALL), and in 2% of patients with newly diagnosed AML [34]. Due to its high presence in most CML patients, it has been described as the major cause of CML. Fusion of BCR to c-ABL inhibits ABL SH3 kinase regulatory domain, resulting in constitutive activation of the ABL tyrosine kinase in BCR-ABL [34]. This kinase activity was shown to be essential for the transforming function of BCR-ABL making this oncoprotein an ideal therapeutic target in CML. BCR-ABL oncoprotein can interact and/or phosphorylate many proteins, making it involved in diverse intracellular signaling pathways to enhance CML survival, inhibit apoptosis, and alter CML progenitor cells adhesion to their external milieu [40, 41]. BCR-ABL oncoprotein overexpression gives the cell the ability to grow in the absence of growth factors, unlike normal cells that undergo apoptosis in such case. This growth factor independency was shown to be accompanied by Bcl2 induction leading to cell resistance to apoptosis and eventually tumor growth [42]. Indeed, BCR-ABL impairs apoptosis via activation of several signaling pathways such as Phosphoinositide 3-kinase (PI3K)-Akt pathway, Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathways [41] and Ras signaling pathway (Figure 5).

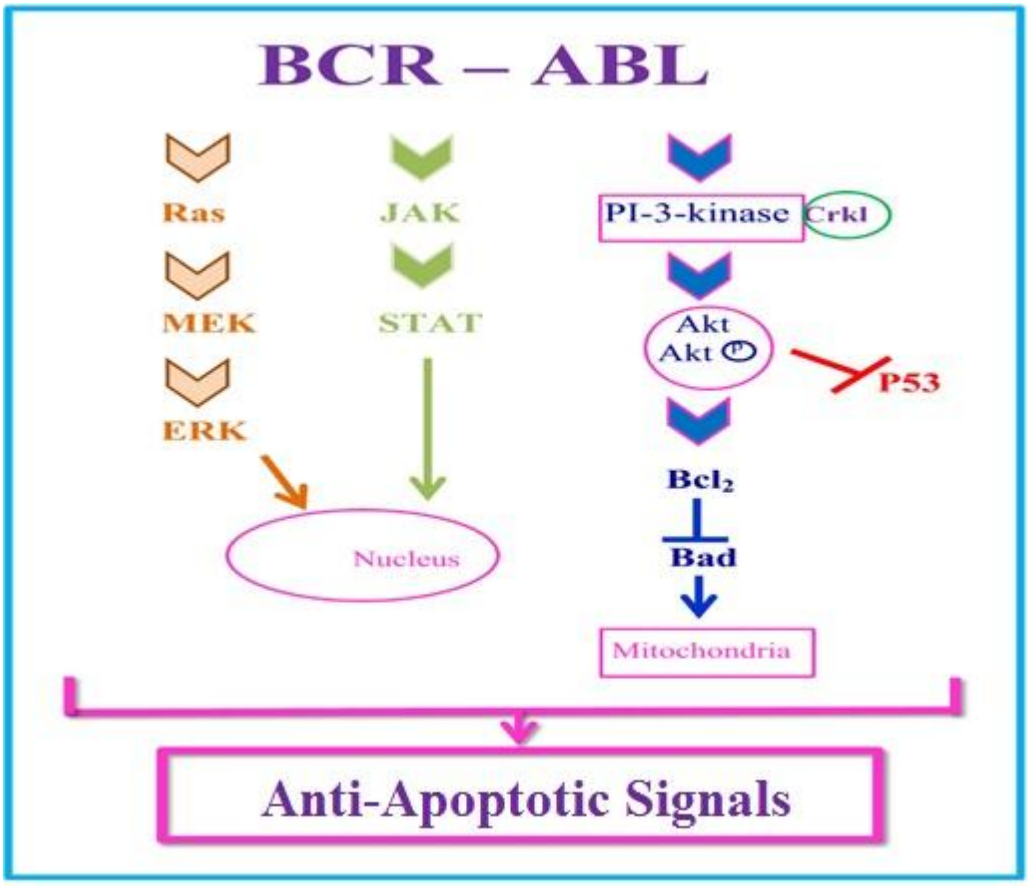


Figure 5: Major signaling pathways stimulated by BCR-ABL oncoprotein in CML cells.

In CML cells, PI3K-Akt, Ras-MEK-ERK and JAK-STAT signaling pathways can be activated by BCR-ABL oncoprotein, even in the absence of IL3. Activation of these pathways leads to cell survival.

Moreover, normal haemopoietic progenitors are in close contact with stroma cells and interact with their bone marrow microenvironment via integrin mediated adhesion [43], which negatively regulates their proliferation [44]. However, the cellular adhesion of CML progenitor cells to their bone marrow microenvironment, and their integrin-mediated adhesion with the extracellular matrix proteins such as fibronectin, is altered by BCR-ABL overexpression. In fact, BCR-ABL oncoprotein is thought to disturb the function of integrin receptors, thus releasing the haemopoietic progenitors from their milieu within the bone marrow [45]. Besides, the cells overexpressing BCR-

ABL oncoprotein are thought to have an altered actin cytoskeleton structure, and increased phosphorylation of certain focal adhesion proteins such as paxillin [46] hence altering the CML progenitor cells' adhesion to their external microenvironment [47]. In summary, BCR-ABL oncoprotein is a constitutively activated tyrosine kinase that promotes cell survival, inhibits apoptosis, transforms haemopoietic cells into growth factor independent cells and alters cellular adhesion, and therefore is the driving force of leukemogenesis of CML.

C. History of Treatment

1. Conventional Therapies (Pre-tyrosine kinase inhibition era)

a. Arsenic

Arsenic has been used in medicine for more than 2400 years for a variety of illnesses including ulcers, the plague, and malaria [48]. In the last century, Fowler's solution, which was developed by Dr. Thomas Fowler in mid- 1700s using arsenic derivatives, was administered to patients suffering from leukemia. The active ingredient in Fowler solution was probably potassium arsenite [36, 49]. In the first documented attempt at therapy, Heinrich Lissauer reported, in 1865, that arsenous oxide was effective in the treatment of CML. In the modern era, interest in arsenic as a chemotherapy was rekindled after it was identified as an active ingredient in traditional medicines in China [50]. In the 1930s, Fowler's solution was used in combination with radiotherapy to treat CML [51]. In the 1950s, this solution was replaced by busulfan [52-54].

b. Radiotherapy

During 1950s, radiotherapy was emerged as the main treatment for CML patients, in which the splenic area or the whole body was exposed to radiations. Unfortunately, such treatment did not achieve enhanced overall survival of CML patients [55].

c. Busulfan and Hydroxyurea

Busulfan is a cancer drug that was first employed to treat CML patients in 1950s. In 1999, busulfan was approved by the US Food and Drug Administration (FDA) for treatment of CML [56]. Busulfan was able to maintain partial hematologic response in most CML patients with serious side-effects including pulmonary fibrosis and seizures [55, 57]. Busulfan was considered the major chemotherapeutic treatment of CML until it was replaced, recently, by imatinib, though it is still used to a certain extent as a result of its relative low cost.

Hydroxyurea, an inhibitor of DNA synthesis and cell cycle, was introduced to treat CML in the 1970s [58]. It was able to control red blood cell count and achieve complete hematologic response (CHR) in 50-80% of CML patients, but cytogenetic responses were rare [59, 60]. Moreover, hydroxyurea was shown to be effective in CML patients resistant to busulfan treatment [58]. However, both busulfan and hydroxyurea were not capable of blocking disease progression into blast crisis and the median survival remained between 3 to 6 years [55].

d. Allogenic Bone Marrow Transplantation

In 1980s, allogenic bone marrow transplantation was introduced as the only curative treatment available, for CML patients [1]. However, recent studies show that only 30 to 70% of CML patients achieved long-term disease-free survival after allogeneic bone marrow transplantation [55], and suffered reoccurrence of Ph^+ leukemia, probably due to the persistence of sufficient amount of Ph^+ progenitor cells in the infused marrow causing the disease relapse [61].

Allogenic bone marrow transplantation is associated with many possible complications and significant cost of mortality caused by graft-versus-host disease and infections [60]. Moreover, it is only suitable for about 20% of CML patients due to limitations related to age, fitness and matched donor availability [62].

e. Interferon – Alpha

In the 1980's, Interferon-alpha (IFNα), a human cytokine became the first line of therapy for CML patients who were not eligible for Allogenic bone marrow transplantation [63]. IFNα is a biologically active molecule produced by immune and non-immune cells in response to viral and microbial infections [64] and to other stimuli including recognition of tumor cells [65, 66]. IFNα has long been used as a therapy for cancer, particularly for CML, owing to its growth inhibitory and immune modulatory activities. In approximately 30% of CML patients, IFNα induces a significant reduction of the malignant Ph-positive cell clone, which apparently prevents the progression to the terminal blast crisis [67, 68]. Although, IFNα was shown to induce hematologic and cytogenetic remissions in patients with chronic phase CML [68], it fails to induce long-

term cytogenetic remissions in most patients [69] and resulted in some intolerable side effects in older patients [55].

2. Tyrosine Kinase Inhibitors (TKI)

a. First Generation TKI - Imatinib

Understanding the pathophysiology of CML has facilitated the development of targeted agents, such as tyrosine kinase inhibitors (TKI) [33]. Imatinib mesylate (imatinib), an orally available TKI, is the first example of these targeted therapies that was approved as the first line therapy in CML with mild to moderate side effects. Imatinib binds to ATP binding-pocket of BCR-ABL TK in its inactive conformation (Figure 6). Imatinib binds to BCR-ABL TK domain, and controls CML progression by preventing the phosphorylation of downstream substrates, thus decreasing cell proliferation and eventually, inducing a complete cytogenetic response in more than 80% of patients with CML in CP.

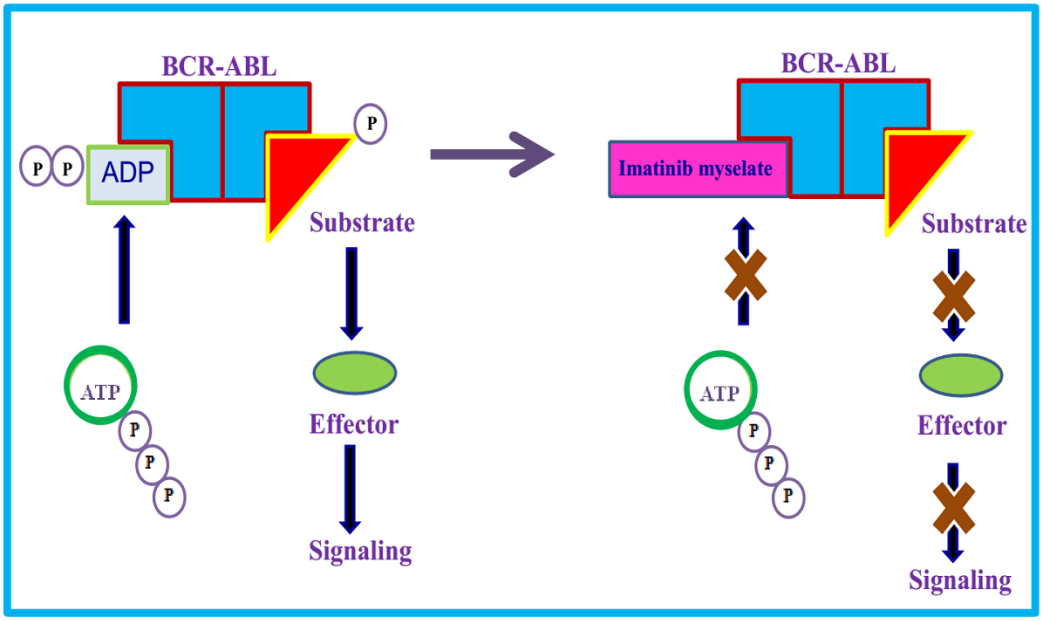


Figure 6: Mode of Action of Imatinib.

Tyrosine kinases have a binding site for ATP. The enzymatic activity catalyzed by a tyrosine kinase is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrates, a process known as protein tyrosine phosphorylation. Imatinib works by binding close to the ATP binding site of BCR-ABL fusion protein. This prevents substrate phosphorylation and signaling leading to inhibition of cell proliferation.

In June 1998, Imatinib was taken into a phase I clinical trial with CP CML patients who failed IFNα treatment. Around 98% of the patients achieved complete hematological response (CHR) within four weeks of treatment. Side effects with imatinib treatment were mild, including nausea, myalgia, edema, and diarrhea [70]. Based on these clinical trials, daily dose of 400mg imatinib was recommended for future studies.

At the phase II clinical trial, imatinib was administered orally at 400mg daily to CP CML patients who had failed IFNα treatment [71]. About 95% of the patients achieved CHR and 60% achieved major cytogenetic response (MCgR) [71]. Moreover, AP and BC CML patients treated with imatinib achieved higher response rates and

longer survival time than patients treated with any of the conventional treatments [72, 73].

In June 2000, the phase III International Randomized Study of Interferon and STI571 (IRIS) study was initiated with newly diagnosed CP CML patients and compared imatinib treatment with IFNα plus cytarabine [74, 75]. After 18 months follow up, 76.2% patients treated with imatinib achieved complete cytogenetic response (CCgR), while only 14.5% patients treated with IFNα plus cytarabine achieved the same response [74, 75].

Despite these remarkably successful results of clinical trials with imatinib, the probability of imatinib to cure CML was questioned continuously, specially that the disease reoccurs in CML patients once imatinib treatment is stopped [76, 77]. Moreover, residual leukemia usually remains detectable by quantitative real time PCR (Q-RT-PCR) in 95% of imatinib treated patients who achieved CCgR [78]. Studies elucidated the presence of a small population of primitive quiescent leukemic stem cells insensitive to imatinib that sustain the disease and provide a reservoir of leukemic cells (Figure 7) [79-81]. On the other hand, imatinib treatment was challenged for other reasons besides its inability to completely eradicate leukemic stem cells and CML eventually. For instance, some CML patients are said to be resistant to imatinib, since once they are treated with imatinib, they are incapable of achieving a complete hematological response, or even a complete cytogenetic remission [74]. In addition, some other CML patients treated with imatinib and achieving a CHR or CCR, or both, eventually stop responding to imatinib treatment. These cases are often explained by an acquired imatinib resistance during the patient's exposure to the drug [82]. Besides, about 40% of these patients acquire imatinib resistance associated with the acquisition

of BCR-ABL KD (BCR-ABL Kinase Domain) mutations [83]. About 60% of these mutations are those at the residues glycine 250, tyrosine 253, glutamic acid 255, methionine 351, phenylalanine 359 [84], and T315I, known as the gatekeeper residue, which is the most troublesome mutation among all mutations [85].

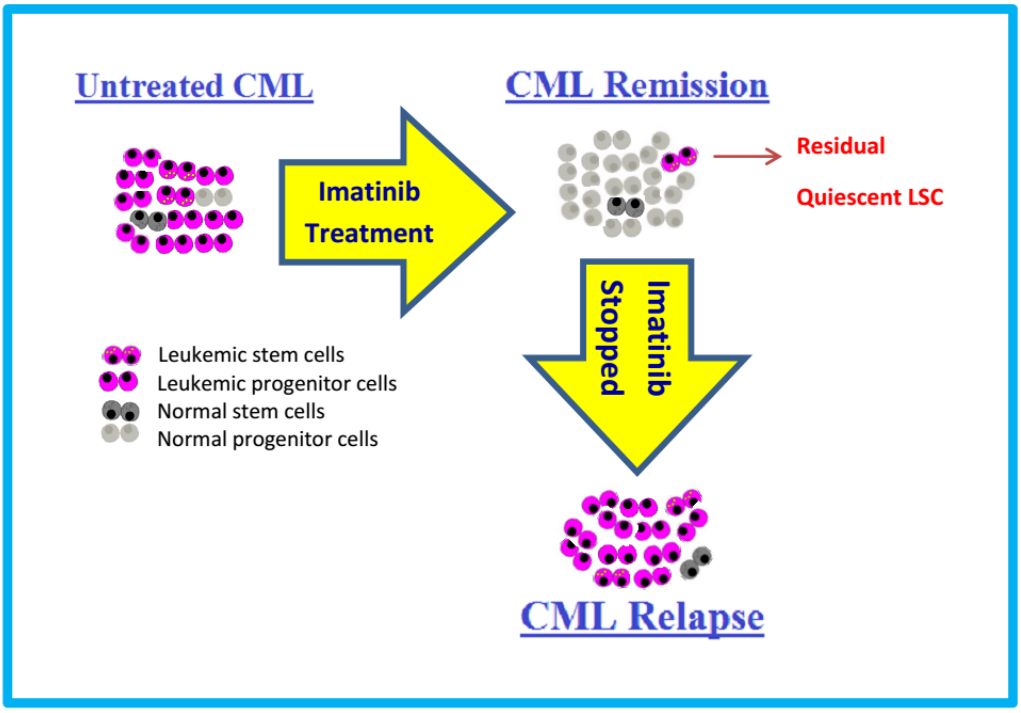


Figure 7: Imatinib achieves long term disease control rather than eradicating CML.

Imatinib induces remission in CML patients but does not eliminate leukemia stem cells (LSCs), which remain a potential source of relapse.

b. Second Generation TKI

With limited follow-up compared to imatinib, the second generation TKI, nilotinib and dasatinib provide several advantages. Response milestones are achieved sooner and in a greater proportion of patients. In addition, these drugs have shown better overall survival [86, 87], and reduced progression to advanced disease [88, 89].

i. Nilotinib

Nilotinib (formerly AMN107) is a second-generation TKI that binds only to the inactive conformation of BCR-ABL oncoprotein, and blocks its activity [90, 91]. It is more potent in binding the BCR-ABL ATP binding site and has a 20 to 50 times better inhibitory activity compared to imatinib [90, 92, 93]. The recommended dosage is 400 mg twice daily, and it is taken orally. In a study conducted by Rosti et al. [94], newly diagnosed chronic phase CML patients treated with nilotinib showed a CCyR in 96 % of the cases. Nonetheless, clinical studies have also demonstrated the effectiveness and safety of nilotinib in imatinib resistant or intolerant CML patients [95, 96], leading to the approval of nilotinib by FDA, in 2007 as a second line treatment option, for treating adult Ph^+ CP and AP CML patients who are resistant to prior therapy including imatinib, and subsequently newly diagnosed adult Ph^+ CP CML patients. Notably, with the exception of the T315I BCR-ABL mutation, nilotinib targets imatinib resistant cases [93, 95, 97], but does not target quiescent CML stem cells [92].

ii. Dasatinib

Dasatinib is a dual Src /Abl tyrosine kinase inhibitor [98]. Dasatinib is an orally administrated drug and is 325-fold more potent than imatinib in cells expressing wild-type BCR-ABL oncoprotein [93]. Almost all BCR-ABL KD mutations can be inhibited by dasatinib except the T315I mutation [93, 99]. In contrast to imatinib and nilotinib, dasatinib binds to both active and inactive conformations of ABL kinase, rendering with much higher binding affinity than imatinib [99]. Clinical trials demonstrated the efficacy and tolerability of dasatinib in all phases of imatinib resistant or intolerant

CML patients [100-102]. Dasatinib was approved by FDA for treatment of adult Ph^+ CML and ALL patients who are resistant to prior therapy including imatinib, as well as newly diagnosed adult Ph^+ CP CML patients. However, dasatinib is not able to eliminate the primitive quiescent CML stem cell population [103], and accordingly like imatinib and nilotinib, dasatinib is unable to cure CML.

c. Third Generation TKI

i. Ponatinib

Ponatinib (AP24534) is an orally high-affinity, pan BCR-ABL TKI with the unique property of inhibiting BCR-ABL T315I [104] which confers resistance to first and second generation TKI. Clinical trials revealed that ponatinib have an anti-leukemic activity in CML patients, including those with BCR-ABL T315I mutation, although responses in patients with BP CML or Ph^+ ALL are typically temporary [105, 106]. However, early analysis evidenced high occurrence of life threatening side effects such as cardiovascular disorders, causing discontinuation of these trials [107].

After a hold due to safety concerns pertaining to vascular occlusion events, regulatory approval in the United States was reinstated for patients with refractory Ph^+ leukemia harboring BCR-ABL T315I mutation or for whom no other TKI is indicated [108]. Although there are evidences suggesting the potential efficacy of ponatinib against all ABL mutations, there is a recent evidence demonstrating the emergence of ponatinib-resistant compound mutants, with more specifically, T315I inclusive compound mutants which confer resistance to all TKIs [109].

d. Mechanisms of TKI resistance

i. BCR-ABL Independent Resistance

- Drug Availability

From a clinical perspective, in order for a drug to be effective it is required to reach its target. Early studies on the pharmacokinetics of imatinib showed considerable interpatient variability in imatinib concentrations [110, 111]. Following ingestion, oral bioavailability of imatinib is established by gastrointestinal absorption and first-pass drug metabolism in the liver, where imatinib is largely metabolized through the cytochrome p450 system. Intrinsic variability of the enzymes' activity between individuals [112], and co-medication that may influence their activity, might explain, the variability in imatinib concentrations.

In addition, imatinib is approximately 95% bound to plasma proteins, mainly to alpha-1 acid glycoprotein (AGP) [113]. Thus, AGP level in plasma may interfere in the availability of free or active drug, thus in its therapeutic effect. Moreover, drug transporters can potentially play a part in resistance to drugs. The interpatient variability in drug availability may be altered either via increased efflux of the drug or decreased influx. Both cases may attribute to the decrease in the intracellular concentration of the drug, thus decreasing its efficacy.

- Clonal Evolution

Clonal evolution refers to CML cells that acquire additional chromosomal abnormalities and epigenetic modulations including methylation [114] and post translational acetylation [115]. Remarkably, such mutations can render the cells more resistant to TKI. Lahaye and colleagues [116] showed that failure to achieve CHR, or

even loss of previous CHR was more likely to be associated with clonal evolution than with ABL kinase-domain mutations. This finding was most obvious in blast crisis, with 73% of patients having clonal evolution compared with 30% with ABL-kinase domain mutations [116].

- CML Stem Cells

Despite the ability of TKI to induce rapid hematologic and cytogenetic responses in the majority of CP CML patients, they fail to eradicate quiescent and primitive CML stem cells (SC), even at high concentrations [99]. Indeed, Valent, P. et al [117], resumed that treatment failure in certain settings has been attributed to the presence of cancer stem cells, which are intrinsically highly resistant to many therapeutic approaches. CML SC, which form 0.5% of leukemic cells, are shown to be unaffected by TKI, probably due to their quiescent nature. Hence, these cells act as a reservoir of the disease causing disease relapse eventually [79-81].

Recent studies revealed that CML SC can survive independently of BCR-ABL kinase activity, since they were capable to proliferate, normally, despite their long-term kinase inhibition [61, 118]. These results suggested that CML SC might not be oncogene addicted, or at least not to oncoprotein kinase activity.

On the other hand, TKIs seem to trigger some critical pathways in CML stem cells stimulating an anti-proliferative activity in these cells, enhancing their quiescent state and therefore rendering them more difficult to eradicate [119-121]. This might explain the insensitivity of CML stem cells to TKI and why these cells persist in patients.

In fact, the presence of CML SC adds an extra challenge in treating CML patients because it creates a new target to hit and eliminate. Alternative targeted therapies will be needed, either alone or in combination with TKI, to suppress CML SC. The identification of CML SC signaling pathways essential for their survival is necessary and may be considered as a major potential target for CML therapy.

ii. BCR-ABL Dependent Resistance

- Increased Expression of BCR-ABL

Amplification of bcr-abl oncogene was first reported in three of 11 patients with acquired resistance [123]. However, in a subsequent study of 66 imatinib resistant patients, only 2 patients showed bcr-abl oncogene amplification [124]. Hence, clinical resistance is much more likely to be due to another mechanism besides bcr-abl amplification. Several studies relate TKI resistance to ABL kinase domain mutations [82].

- ABL Kinase Domain Mutations

The most frequently described mechanism of acquired resistance to TKIs is the occurrence of point mutations in ABL kinase domain. Such mutations cause either the impairment of TKI binding to BCR-ABL oncoprotein or the inhibition of BCR-ABL by assuming the inactive conformation appropriate for TKI binding. The emergence of mutations within the kinase domain of BCR-ABL is regularly associated with resistance to TKI therapy [125]. Over 100 BCR-ABL kinase domain point mutations have been linked with clinical imatinib resistance [82]. To overcome the resistance, second generation TKIs have been developed. However, these treatments may lead to new

BCR-ABL mutations. The presence of existing mutations after imatinib failure, as well as development of new mutations on a subsequent second TKI is naturally a potential source of resistance to successive TKI [126-129]. Among all mutations on ABL Kinase domain, the most famous mutation T315I, confer resistance to both first and second generations TKI [126, 130, 131]. T315I mutation accounts for 15–20% of mutations of the ABL kinase domain [132].

- T315I Mutation

In 2001, Gorre and co-workers [123] described 11 patients treated with imatinib for CML blast crisis or Ph-chromosome-positive acute lymphoblastic leukemia (ALL) who relapsed on treatment. Bcr-abl gene amplification was only detected in three patients. Sequencing of the ATP-binding pocket and the activation loop of the kinase domain showed an identical cytosine to thymidine mutation at ABL nucleotide 944 in six of nine assessable patients. This mutation resulting from the substitution of threonine residue, called gate keeper contact region, with an isoleucine residue at an amino acid position 315. Notwithstanding the TKI remarkable activity against most BCR-ABL mutants, neither first nor second generation TKI are active in patients who carry the T315I mutation [133, 134].

- **Why Is T315I So Difficult to Target With ATP-Mimetic TKIs?**

T315 locates at the periphery of the nucleotide-binding site of ABL kinase within the hinge region of the enzymatic cleft, stabilizes imatinib binding through hydrogen-bond interactions, and regulates access to a deep hydrophobic pocket in the active site [85, 135]. It is noteworthy that, although imatinib and nilotinib bind BCR-

ABL kinase in the inactive conformation and dasatinib does so in both, active and inactive conformation, all three TKIs make a critical hydrogen bond with the side chain hydroxyl group of T315 [85, 97, 99]. A mutation of the threonine gatekeeper residue to isoleucine prevents the formation of this critical hydrogen bond. Second, mutations may cause steric hindrance between the large hydrophobic isoleucine residue and any of the TKI thus blocking the access of the latter to the hydrophobic pocket in the proximity of T315, and inhibits its action [123]. Third, T315 participates in a network of hydrophobic interactions when the kinase is in the active conformation. Its mutation to isoleucine promotes the assembly of an enzymatically active kinase conformation through the stabilization of a series of hydrophobic interactions [136, 137]. Consequently, T315I mutation results in complete insensitivity to imatinib, nilotinib, dasatinib [123, 138-140]. An array of agents with significant preclinical activity against BCR-ABL T315I positive cells is currently undergoing clinical trial testing in patients who have failed imatinib and other TKs [141]. Some of these agents are potent inhibitors of aurora kinases and have also been found to be active against the BCR-ABL T315I cells. In summary, the most famous mutation, T315I mutation, confers resistance to all approved TKIs except ponatinib, which has toxicity limitations [109].

D. Novel Combination – Arsenic Trioxide and Interferon Alpha

1. Arsenic Trioxide (ATO) Mode of Action

Arsenic trioxide has been used as a medical therapy for over 2,400 years. Its use as an anti-leukemic agent dates back to the late 19th century, and it was only replaced by modern chemo-therapeutics in the 1950s. In fact, ATO has shown promise in many hematopoietic malignancies. ATO has shown its potent antitumor

effects in in-vitro and in-vivo studies, but its precise mechanism of action is still not well understood. Below are stated some of the different mechanisms by which ATO promotes cell death of target cells.

a. ATO induces apoptosis

ATO-induced apoptosis involves the change of mitochondrial membrane potential, thus modulating expression of apoptotic proteins including cytochrome c, which is normally sequestered between the mitochondrial inner and outer membranes, to be released into the cytosol. Cytochrome c, then, binds and activates Apaf-1, which in turn activates procaspase-9 [142-144]. Caspase-9 degrades procaspase-3, and the downstream substrates of caspase-3, that results in the cleavage of caspase-activated DNase, hence DNA fragmentation eventually [145] (Figure 8). ATO-induced apoptosis was also shown to be accompanied by the accumulation of reactive oxygen species (ROS). ATO has the ability to bind to antioxidant enzymes, such as thioredoxin reductase via its sulfur selenium group [146], and inhibits their action, thus increasing the cellular levels ROS, leading to cell death.

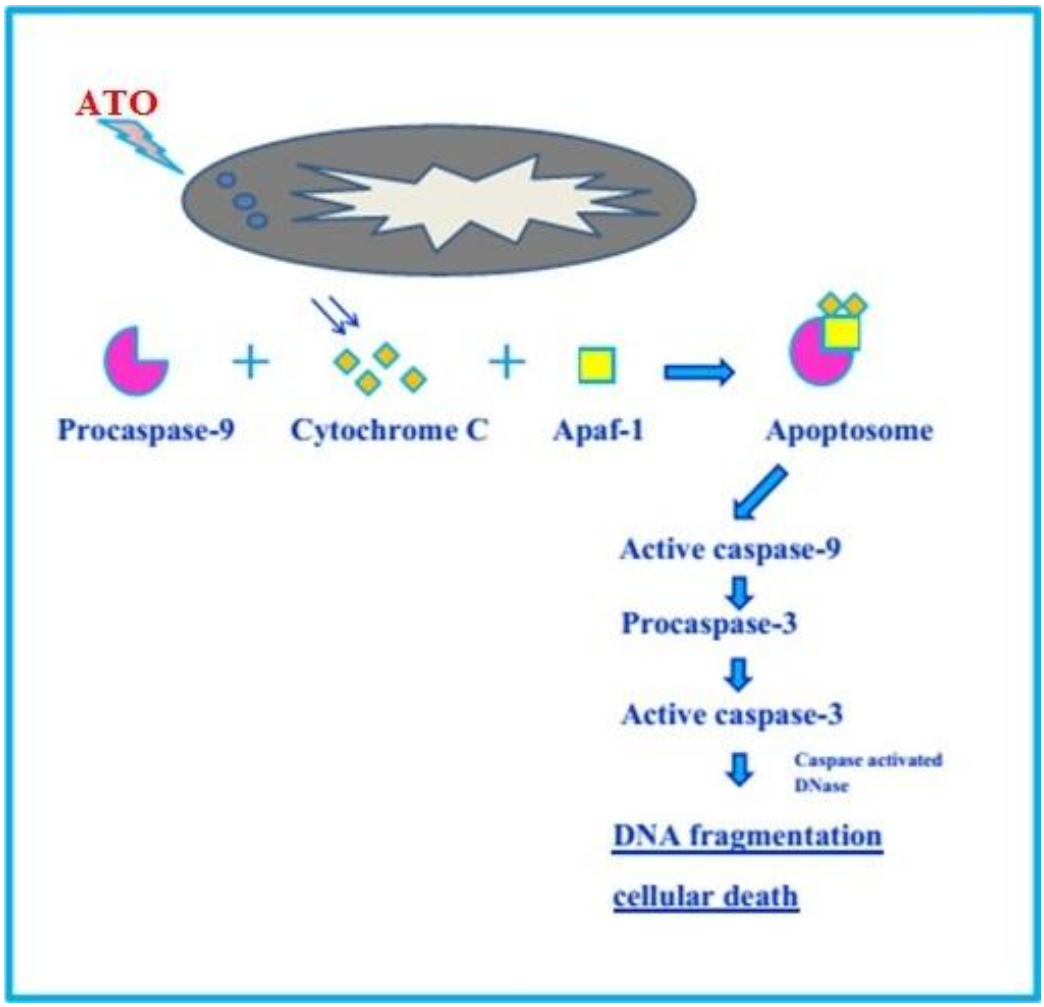


Figure 8: ATO induces apoptosis.

ATO causes the release of cytochrome c, a pro-apoptotic molecule into the cytoplasm. Cytochrome c forms the apoptosome complex with pro-caspase 9 and APAF-1. Procaspase-9 is then cleaved forming an active caspase-9 that is followed by activation of caspase-3 and other downstream caspases. Finally, caspase-3 cleaves caspase-activated DNase and causes DNA fragmentation.

b. ATO inhibits NFκB

NFκB is a transcriptional factor promoting cytokine production and cell survival with an important role in many cancer cells. Activation of NFκB depends on the integrity of the IκB kinase (IKK). In the cytoplasm, IKK phosphorylates IκBα that is NFκB inhibitor, leading to its subsequent ubiquinitation and degradation, causing the release of NFκB and its translocation to the nucleus. In the nucleus, NFκB turns on the expression of proliferative and anti-apoptotic genes. NFκB pathway was shown to be inhibited by ATO in cancer cells at different levels. ATO binds to cysteine-179 in the activation loop of the IKK catalytic subunit and leads to its inhibition [147]. Subsequently, IKK will no longer be able to phosphorylate IκBα, thus sequestering NFκB in the cytoplasm where it will not be able to activate genes required for cell proliferation and evasion of apoptosis (Figure 9).

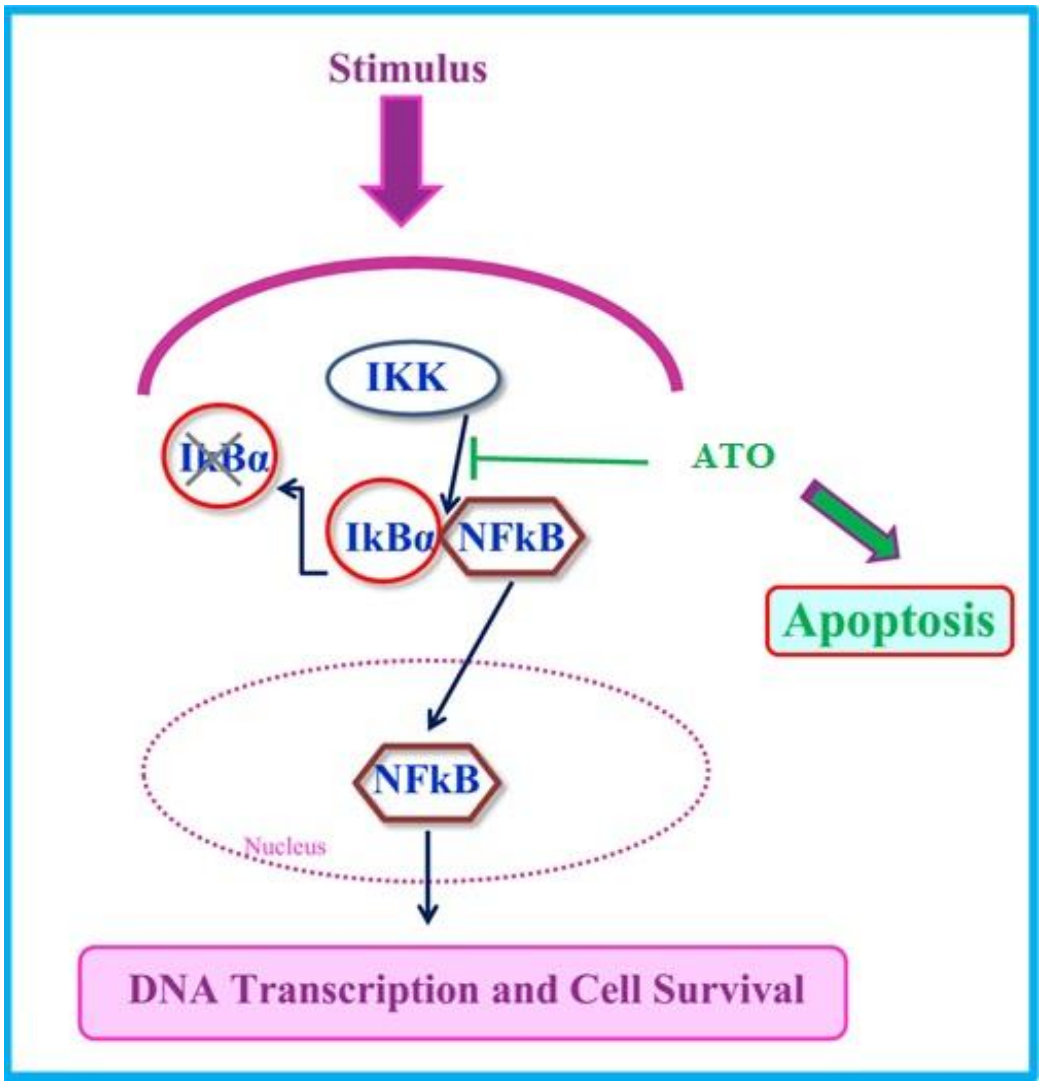


Figure 9: ATO inhibits NFκB translocation to the nucleus.

IKK activation catalyzes the degradation of IκBα, leading to the release of NFκB. NFκB can then translocate to the nucleus where it regulates the expression of genes leading to cell survival. However, ATO blocks the activity of IKK leading to inhibition of NFκB – dependent genes transcription.

c. ATO degrades PML-RAR oncoprotein

Promyelocytic leukemia protein (PML) is a protein encoded by pml gene. PML localizes to nuclear bodies and functions as a tumor suppressor protein, where it controls processes including apoptosis, cellular proliferation, and senescence [148, 149].

APL (Acute promyelocytic Leukemia) pathogenesis is associated with a chromosomal translocation generating PML-RARα fusion protein plays a major role in disrupting the apoptotic function of PML protein. Studies show that ATO binds directly to PML and prompts its sumoylation with small ubiquitin-like modifier SUMO1 and its subsequent ubiquitination, thus, triggering proteosome-dependent PML-RARα degradation [150], hence allowing the wild-type PML to re-form nuclear bodies, thus activating PML pro-apoptotic functions through different mechanisms. In fact, ATO that triggers the PML-NB reformation, causes the activation of p53 retinoblastoma tumor suppressor and hence, the induction of cellular death [151, 152] (Figure 10).

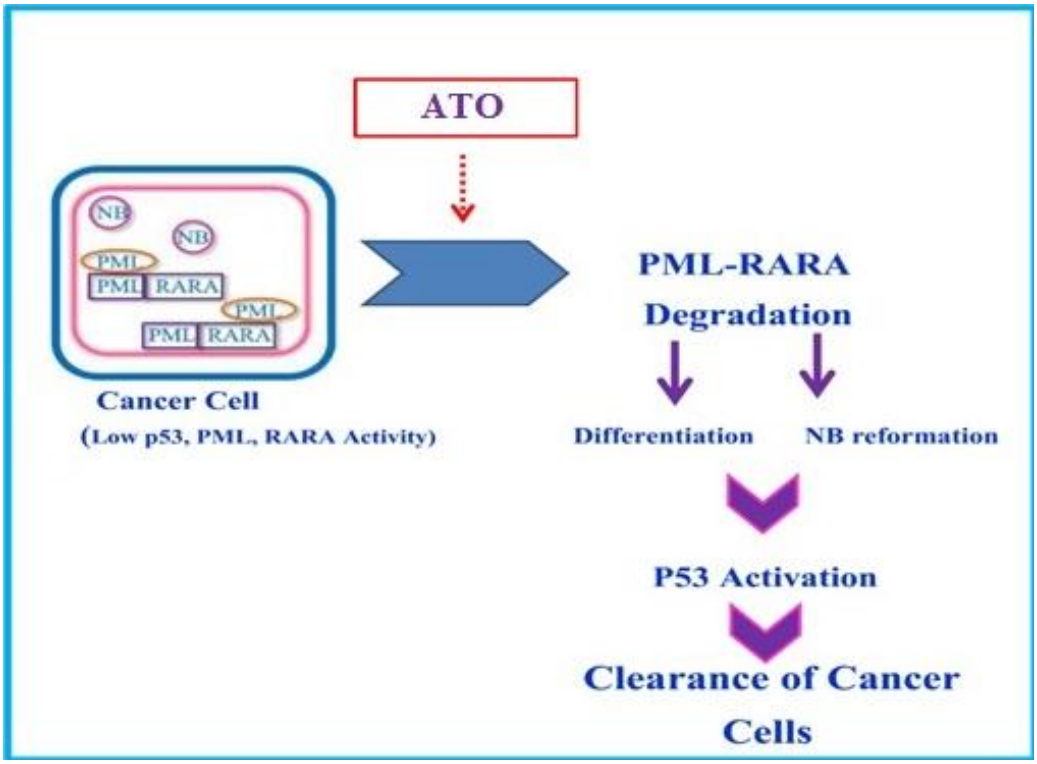


Figure 10: ATO triggers cellular death in APL cells via PML-RARA degradation and p53 activation.

ATO degrades the oncogenic fusion protein PML-RARA, allowing wild-type PML to re-form nuclear bodies, activate p53, thus inducing cellular death.

Moreover, ATO promotes the accumulation of Daxx, which acts mostly as a transcriptional corepressor. Daxx represses the expression of several anti-apoptotic genes when localized in PML-NBs, and activates the transcription of pro-apoptotic genes thereby eliciting pro-apoptotic functions [153]. Thus, PML degradation may be sufficient to trigger Daxx-dependent apoptosis (Figure 11).

Figure 11: Daxx mediates apoptosis by ATO.

Daxx is a nuclear protein that mostly represses transcription and co-localizes with PML in nuclear bodies. ATO promotes the accumulation of Daxx, thus triggering Daxx-dependent apoptosis.

d. ATO inhibits telomere lenghthening

Telomerase is a reverse transcriptase, which maintains telomere length by adding nucleotides to the single-stranded DNA of the telomere during cell division. Telomerase consists of a protein component (hTERT), which is the catalytic subunit of telomerase, and an RNA template component (hTERC). An indirect mechanism by which ATO may promote apoptosis is the inhibition of telomerase activity [154]. This decrease in telomerase transcription may be related to direct effects of ATO on transcription factors such as Sp1 and Myc, or on the expression of hTERT which limits its reverse transcriptase activity [155] (Figure 12). In any case, this results in an increase in genomic instability and chromosomal abnormalities leading to cell death.

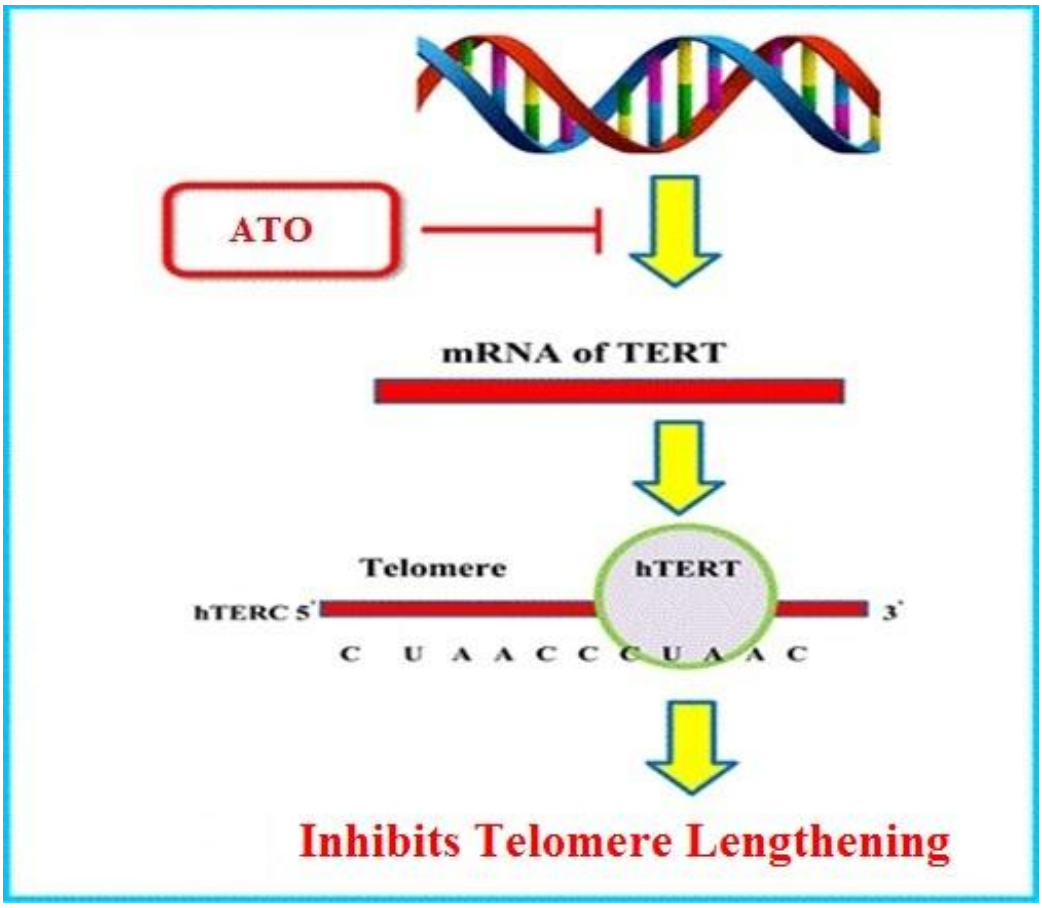


Figure 12: ATO inhibits telomere lengthening.

ATO reduces the expression of hTERT, thus inhibiting the telomerase activity

e. ATO suppresses angiogenesis

Endothelium and angiogenesis play a major role in the growth and proliferation of liquid tumors including leukemia. Studies have shown that ATO caused inhibition of VEGF production in ATL (Adult T-cell Leukemia) cells. These results suggest that ATO exerts antiangiogenic effects by interrupting a reciprocal stimulatory loop between leukemic cells and endothelial cells by inhibiting leukemic cell VEGF production, and causing apoptosis of both cell types and inhibiting leukemic cell VEGF production [156] (Figure 13). This impaired production or expression of VEGF is a major cause for angiogenesis disruption.

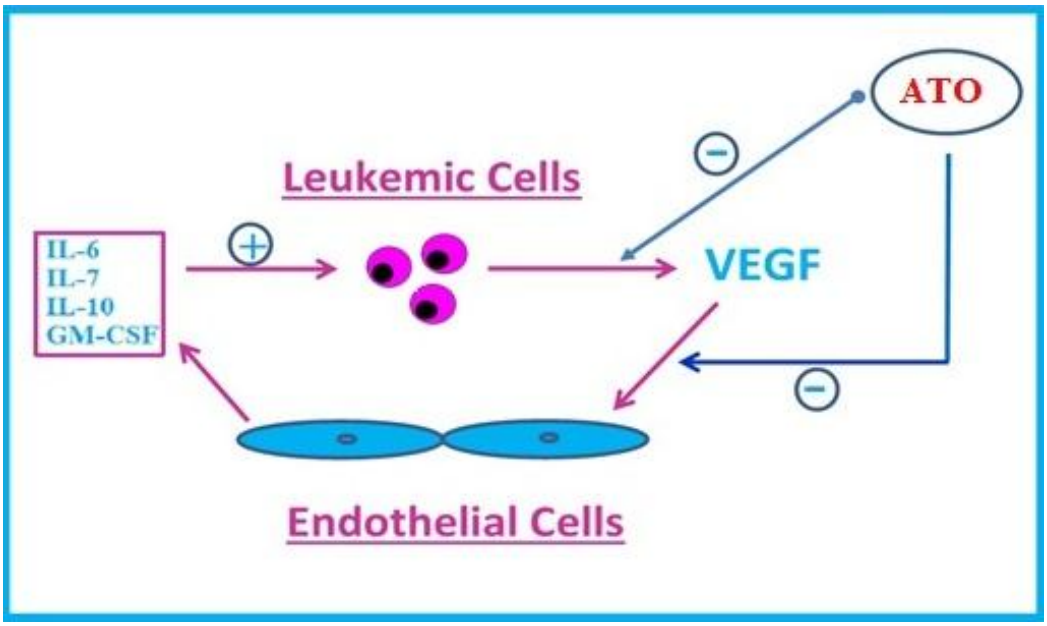


Figure 13: ATO inhibits angiogenesis.

ATO interrupts a reciprocal stimulatory loop between leukemic cells and endothelial cells by causing apoptosis of both cell types and by inhibiting leukemic cell VEGF production.

f. ATO disrupts microtubule assembly and spindle formation

The cytoskeleton has also been suggested as a potential cellular target for ATO that has the ability to bind to the thiol groups in the major constituent of the cytoskeleton, tubulin. ATO markedly inhibits GTP-induced polymerization of monomeric tubulin for microtubule formation in CML cells [157]. Thus ATO promotes apoptosis through the disruption of microtubule assembly and spindle formation during mitosis [157, 158], perhaps via interactions with tubulin and other cytoskeleton elements [159] (Figure 14).

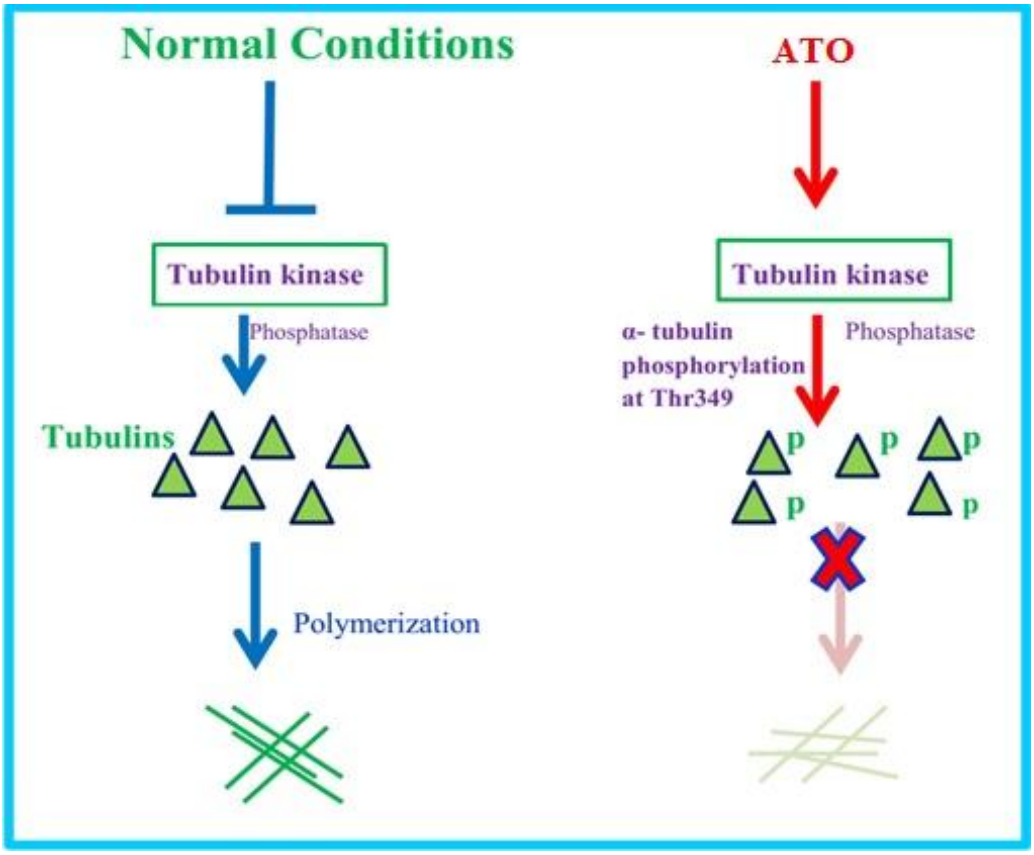


Figure 14: ATO disrupts microtubule formation.

ATO markedly inhibits GTP-induced polymerization of monomeric tubulin for microtubule formation, thus promoting apoptosis.

g. ATO induces autophagy

Autophagy is a cell death mechanism distinct from apoptosis, also defined as type II programmed cell death, involving autophagosomic / lysosomal degradation of cellular components [160]. There is emerging evidence that autophagy plays an important role in the regulation of malignant cell survival [160]. A recent study demonstrated that degradation n of the leukemia-inducing BCR-ABL oncoprotein involves induction of autophagy, and that the autophagic process plays a key and essential role for the generation of the antileukemic effects of ATO [161]. Crucial to

that degradation is the association of the oncoprotein with the poly-ubiquitin binding protein SQSTM1/p62, where ATO treatment promotes formation of SQSTM1/p62-BCR-ABL complexes localized to autolysosomes [161]. In another recent study, autophagy was shown to be activated by ATO in acute myelogenous leukemia cells through the activation of MEK/ERK pathway [162]. Thus ATO seems to have the ability to induce autophagic cell death in leukemic cells (Figure 15).

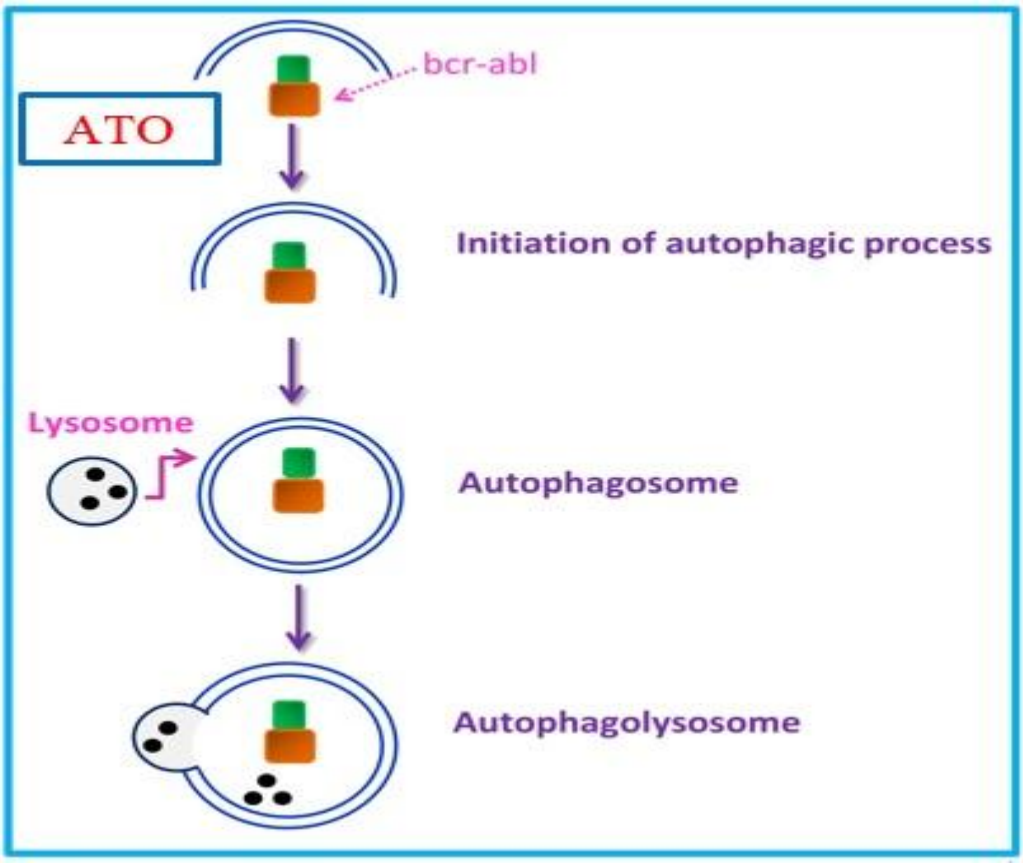


Figure 15: BCR-ABL degradation via the autophagic pathway.

Proposed model for ATO–dependent interaction of p62/SQSMT1 with BCR-ABL and the subsequent autophagic degradation of BCR-ABL.

h. ATO induces CML LSC exit from quiescence

Interestingly, Ito et al [150] has demonstrated that CML patients with low PML expression show a higher CMR and CcyR and a better overall survival rate with respect to those patients with high PML expression. This suggests that in CML, low PML expression is correlated with better predicted clinical outcome.

Moreover, Ito et al [150] by analyzing the PML protein level in murine normal HSC and CML LSC revealed that level of PML protein and the number of PML-NB was high in HSCs compared to committed cells. Similarly, most CML murine samples

expressed high levels of PML blast cells. In addition, using a CML mouse model where genetic inactivation of the pml gene was induced, show that pml-deficient CML LSC became exhausted and unable to initiate CML in transplanted animals [150]. Hence, PML plays a major role in maintaining LSC in a quiescent state. Inhibition of PML by ATO disrupted LSCs maintenance and increased the efficacy of anti-leukaemic therapy by sensitizing LSCs to pro-apoptotic stimuli. In combination with the chemotherapeutic drug Ara-C, ATO increased the efficacy of this anti-leukemic treatment for LSCs, most likely by inducing their exit from quiescence [150].

In addition, another study done in 2008, demonstrated the high efficacy of ATO in combination with RA to eradicate APL LSC through PML-RAR degradation [163]. Significantly, ATO has successfully been used clinically to treat PML- RAR induced APL showing that this drug can be effectively used in patients [50, 164, 165]. Future studies need to determine whether ATO can activate dormant stem cells also in other forms of human leukemia, not driven by oncogenic PML.

2. Interferon – Alpha (IFNα) Mode of Action

The therapy of CML has evolved significantly over the past 2 decades. Before the availability of imatinib, IFNα has been used as a therapy for cancer, particularly for CML, owing to its growth inhibitory and immune modulatory activities. Interestingly, IFNα treatment had a significant impact on the outcome of CML patients [166] and was associated with the achievement of hematologic and even molecular remissions in those patients. However, significant toxicities were frequently encountered causing depression and neurologic disturbances.

IFNα is a cytokine produced by a variety of immune and non-immune cells in response to microbial infections or recognition of tumor cells. Upon engaging their receptors, IFNα activate a set of genes that inhibit viral replication and clear infected cells. However, the mystery of how IFNα influences CML progression is still under investigation. Nevertheless, below stated some of the studied mechanisms of IFNα that may explain its effect on CML patients.

a. IFNα induces cell cycle arrest and inhibits cells growth

In vitro studies, show that IFNα has a tendency to suppress the ability of normal human haemopoietic progenitor cells (HPCs) to form colonies, even in the presence of a variety of cytokine combinations and conditioned media [167-169]. CML HPC compared to normal HPC were even more sensitive to the inhibitory effects of IFNα [170]. Moreover, Peschel and coworkers have also reported that IFNα may have an influence on cytokine expression that is triggered by the bone marrow stromal cells, including granulocyte–macrophage colony-stimulating factor and interleukin-1b [171], and the inhibition of the production of IL-1 receptor antagonist, leading to such myeloproliferative suppression [172, 173].

Bhatia et al [174] have shown that in CML patients where CML progenitor cells adhesion to bonemarrow stroma is defected, IFNα treatment results in the inhibition of CML progenitor cells proliferation and restoration of the normal haemopoietic cell adhesion. Interestingly, the growth-inhibitory effects of IFNα seem to require activation of the mitogen-activated protein kinase p38 in CML progenitors [168, 175]. IFNα treatment activates p38 by phosphorylation, which in turn leads to the transcription of IFNα inducible genes.

In addition, IFNα was shown to induce G1 cell cycle arrest [176, 177]. For instance, the Cdk inhibitors p19 and p21 were strongly up-regulated in mouse machrophages after treatment with IFNα and these binding to the G1 cyclin/Cdk complex lead to reduction of its kinase activities and hence G1 arrest in the early phases of IFNα treatment [178]. Other studies show that IFNα treatment can also induces Cdk inhibitors p15 and p27 [179-181], resulting in cell cycle arrest at the G1 phase. Moreover, the upregulation of p21 and p27 is possibly due to the activation of the IFNα-inducible gene RIG-G [182]. On the other hand, the transcription factor c-myc has been shown to be down regulated in response to IFNα, resulting in cell cycle arrest [183-186] (Figure 16).

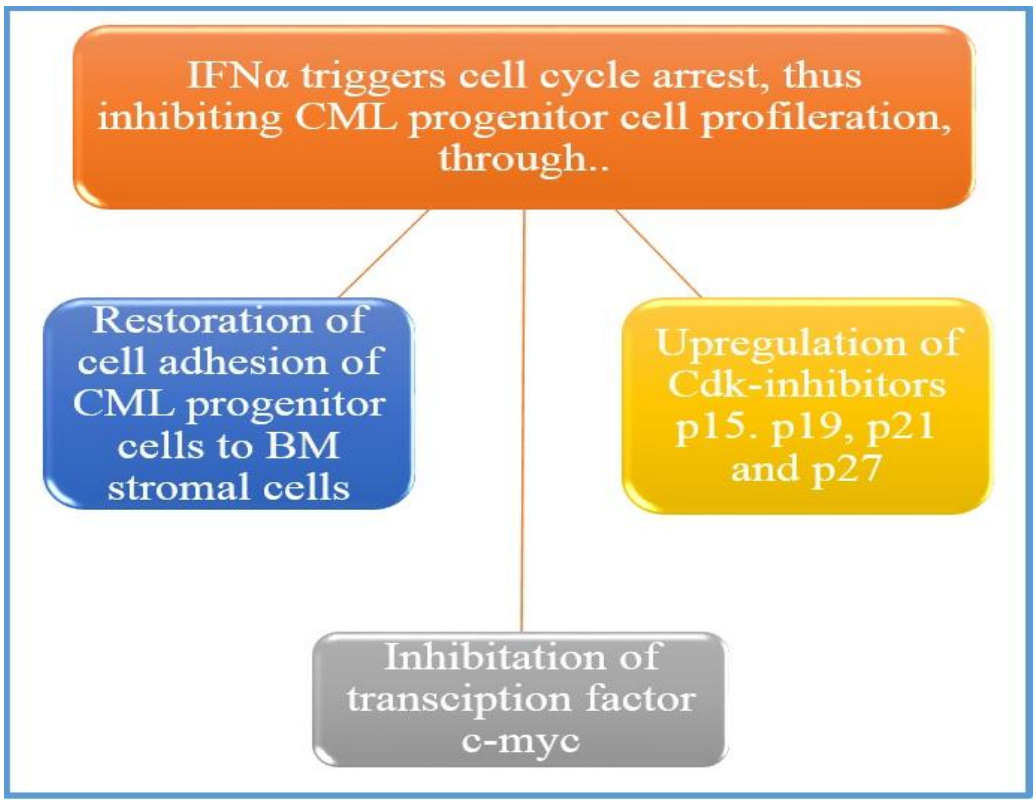


Figure 16: IFNα induces cell cycle arrest.

b. IFNα induces apoptosis:

Treatment with IFNα results in the up regulation of pro-apoptotic proteins such as first apoptosis signal (Fas), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) [187-191]. These proteins can interact with Fas associated death domain (FADD) or TRAIL-receptor proteins, resulting in initiation of apoptosis through activation of caspase 8 [192, 193]. In addition, IFNα treatment can also up regulate caspase 4, initiator caspases 8 and 9, as well as the effector caspase 3 [194, 195]. These proteins lead to an increased sensitivity of the cell to a pro-apoptotic stimuli, causing the mitochondria to release cytochrome c to the cystosol, and activating the remainder of caspase cascade, leading to cell death eventually (Figure 17).

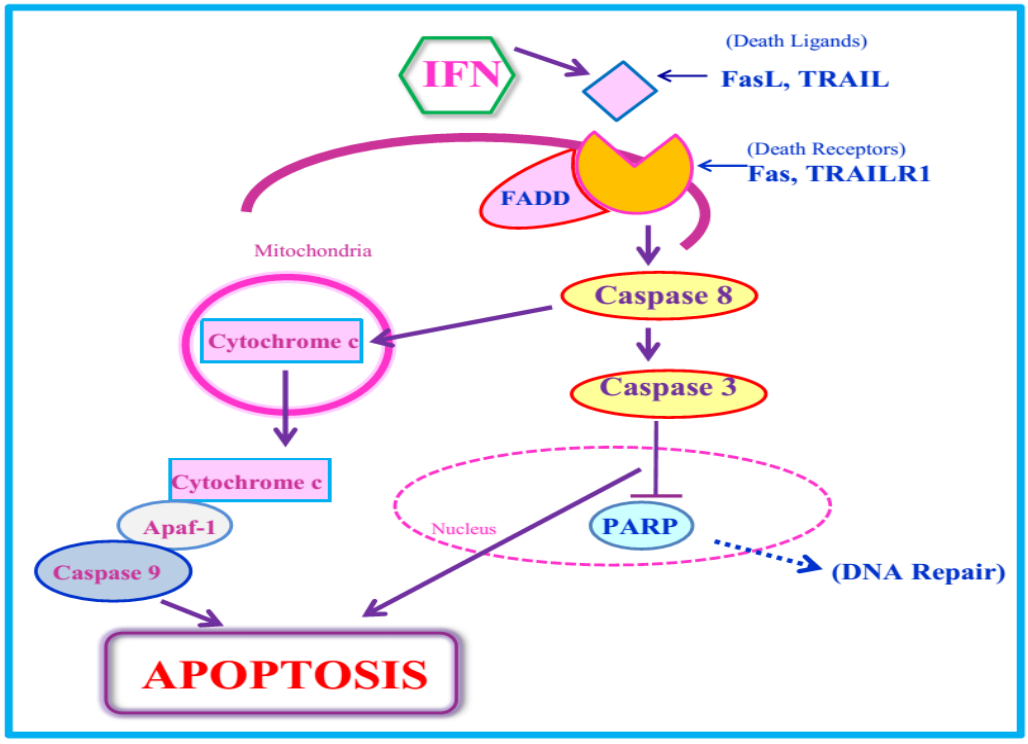


Figure 17: IFNα induces apoptosis.

IFNα induces apoptosis through activating Fas/TRAILR1 leading to the activation of caspase 8 and caspase 3. This causes the mitochondria to release cyctochrome c and initiate caspase cascade leading to apoptosis.

c. IFNα induces CML CTLs and other immune cells

IFNα induces cytotoxic T lymphocytes (CTLs) specific for CML progenitors. In fact, CTLs have been observed in virtually all CML patients who respond to IFNα therapy but not in non-responders and in most patients in remission following imatinib therapy [196-199]. CTLs are capable of killing CML HPCs [197]. Burchert et al [200] have demonstrated that these antileukemic CTLs persist in CML patients who continue to receive IFNα therapy after imatinib therapy is interrupted. Furthermore IFNα treatment leads to continued proliferation of central memory T cells resulting in the expansion of these CTL compartment.

In addition, IFNα is shown to augment the functional activity of T cells, macrophages, and natural killer cells, which may cooperate to target a number of malignancies including CML [201, 202]. Moreover, in both, in vitro and in vivo studies, IFNα treatment causes CML mononuclear cells to differentiate into dendritic cells, which serve as antigen-presenting cells for CML-specific peptides [203, 204]. The dendritic cells that are favored by IFNα is endowed with an ability to capture apoptotic bodies and promote $CD8^+$ T-cell cross-priming, which may provide a possible explanation for the autoimmune reactions and antitumor effects associated with IFNα therapy [204] (Figure 18).

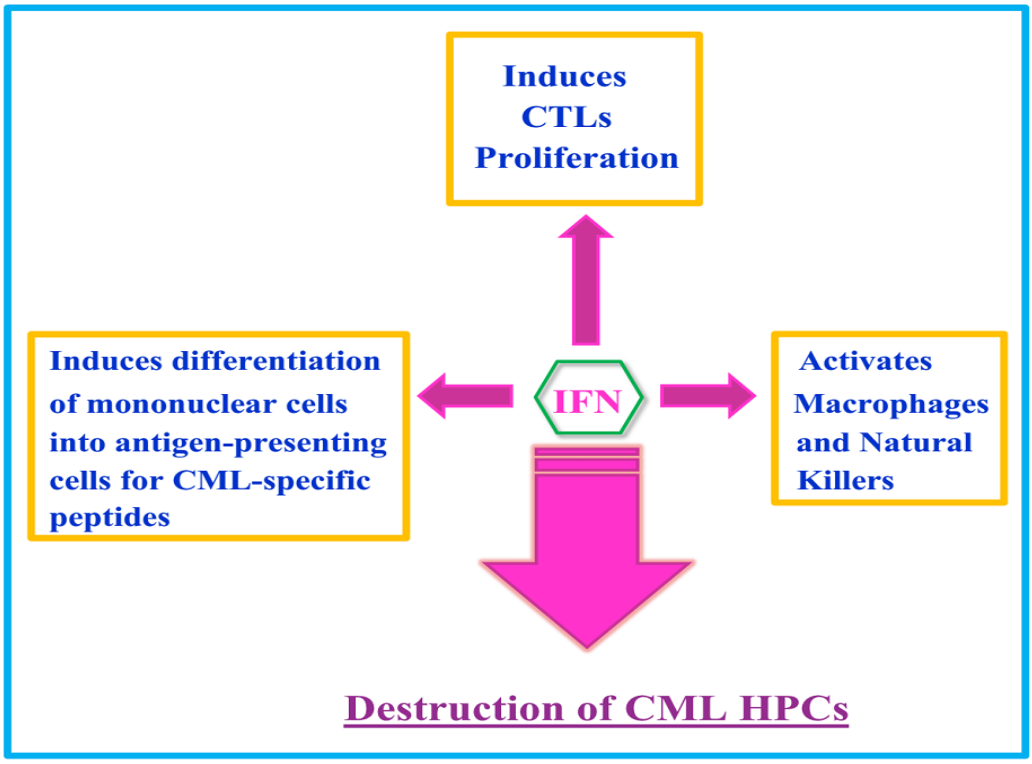


Figure 18: IFNα induces destruction of CML HPC pool.

d. IFNα promotes the cycling of haemopoietic stem cells

Recently 2 groups have independently reported that IFNα has a surprising effect on murine bone marrow HSCs [205, 206]. The first group, Essers et al [205], using a mouse model (C57BI/6 mice) commonly employed in the study of HSC function, found that high levels of IFNα induce murine HSCs to exit from their normal quiescent state and transiently proliferate. The second group, Sato et al [206], were studying the immune modulatory effects of IFNα and other cytokines on immune effector cells when they found that mice lacking a component of the IFNα signaling pathway had an unexpected imbalance of proliferation within the HSC pool. Both groups tracked their observations to a direct effect of IFNα on the proliferation state of HSCs. The point that IFNα may induce the activation of the dormant HSC pool, suggests that IFNα could be

used as a stem cell targeting drug in a combined treatment protocol. Whether IFNα is also able to activate dormant human leukemic stem cells remains to be shown. Nonetheless, long-term high dose of IFNα, the first-line treatment of newly diagnosed patients before IFNα was replaced by imatinib in 2001, had a positive effect on a significant number of CML patients.

Imatinib, as well known, must be taken life-long in most patients, even those with CMR, or else the disease will relapse upon imatinib discontinuation. However, in a French CML trial, a small group of six patients' experienced continued long-term remission even after imatinib was stopped. Strikingly, these six patients had been treated with IFNα before they were switched to Imatinib in 2001 [207]. In contrast, patients from the same study, and from other clinical cohorts, who achieved remission after imatinib treatment but were not previously treated with IFNα, often relapsed upon imatinib discontinuation [207-209]. This indicates that IFNα may have activated and mobilized CML LSCs in these patients, rendering them more sensitive to the subsequently administered imatinib, and thereby allowing eradication of the leukemic clone (Figure 19).

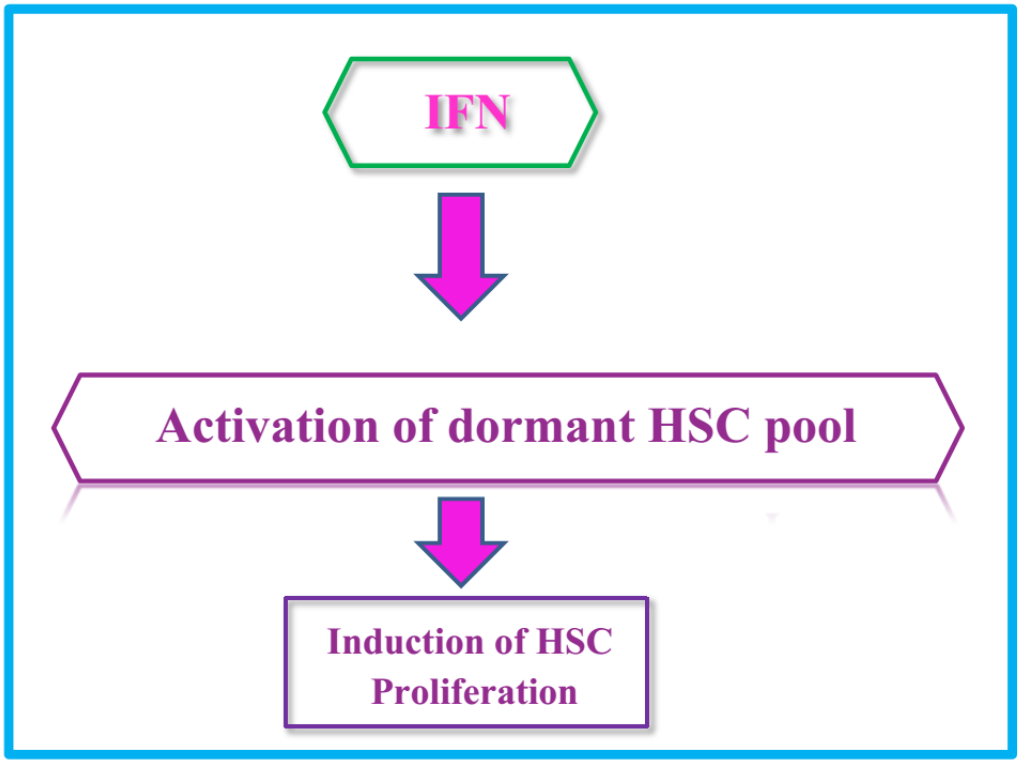


Figure 19: IFNα induces activation of HSC pool thus inducing their proliferation

3. Arsenic Trioxide- Interferon Alpha (ATO/IFNα) Combination

As shown earlier, ATO and IFNα show separate antileukemic activities against either leukemic cells or leukemic stem cells. This fact suggests a possible role for ATO and IFNα combination in eradicating CML LSC and eventually curing CML. In fact, our recent study revealed that ATO and IFNα combination is effective in preclinical models of CML with wild type BCR-ABL. This combination was shown to be superior to imatinib in CML mice [210]. Our study demonstrated that ATO and IFNα combination synergistically inhibited proliferation and activated apoptosis in wild-type CML cell lines. Interestingly, ATO and IFNα combination reduced the clonogenic activity of primary bone marrow cells derived from newly diagnosed CML patients. In addition, this study revealed a significant prolongation in the survival of primary CML

mice treated with ATO and IFNα combination as compared to each drug alone. Importantly, serial transplantation assay showed that the survival of secondary recipients of spleen cells from ATO and IFNα-treated primary mice was significantly prolonged, with one third of the mice not developing the disease. These results highlight the role of ATO and IFNα combination in inhibiting CML LSC activity, unlike imatinib that, as expected, was shown to enrich them. Interestingly, the combination's anti-leukemic activity was BCR-ABL independent since it did not alter neither the level nor the activity of BCR-ABL oncoprotein [210].

On the other hand, previous studies were done to investigate the role of ATO and IFNα combination on ATL cell lines [211]. Results showed that the combination inhibited proliferation and induced apoptosis in HTLV 1 positive cells. ATO and IFNα combination inhibited the NFkB pathway and triggered the degradation of the viral oncoprotein Tax that is essential for the survival of ATL cells [211]. Interestingly, in 2010, El Hajj et al [212] validated that ATO and IFNα work together to cure murine ATL derived from Tax transgenics through selective abolishing of LSC activity. In addition, in 2009, kshour et al [213] investigated the effect of ATO, interferon, and an antiretroviral agent zidovudine in 10 newly diagnosed chronic ATL patients. Impressively, 100 % response rate was detected, including 7 complete response, 2 complete response with > 5% circulating atypical lymphocytes, and 1 partial response. In this report, we investigate the anti-leukemic effect of ATO and IFNα in Ar230-R and K562-R, two imatinib-resistant human CML cell lines, and its anti-tumor activity in CML mouse model harboring the T315I mutation that is resistant to all TKIs.

Chapter II

Methods

A. Culture of CML Resistant Cell Lines

The two imatinib-resistant CML cell lines used in all in vitro experiments are Ar230 and K562 resistant cell lines (Ar230-R and K562-R). These cells were a generous gift from Francois – Xavier Mahon, and were generated by culturing them in presence of increasing concentration of imatinib. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1mM sodium pyruvate, 50 U/ml penicillin-streptomycin antibiotics, and 20µg/ml kanamycin solution. The cells were grown in a humidified incubator (5% CO2) at 37^{o}C. Cells were split every two days to maintain a density of 2 x 10^{5} cell/ml. After splitting, imatinib drug of a final concentration 1µM was added to the media to inhibit the growth of sensitive clones. Imatinib was removed at the time of seeding prior each experiment.

B. Drugs Preparation

1. Arsenic Trioxide (ATO)

Arsenic trioxide (MW 197.841) (Sigma) is provided as a powder that is used to prepare a 100 mM stock solution. 0.1978g of powder arsenic was dissolved in 10 ml of 1 M NaOH and then filtered using a 0.22 µm syringe filter and stored at 4^{o}C. Prior to applying the drug to cells in culture, a fresh 1:1000 dilution is performed in phosphate buffered saline (PBS) to obtain a working concentration of 0.1mM; 5 µl of diluted arsenic is added to each 1 ml of media for a desired final concentration of 0.5µM; 10µl

is added for a desired final concentration of 1µM, and 20µl is added for a final concentration of 2µM.

2. Interferon - Alpha (IFNα)

Recombinant interferon-alpha 2a was provided (Roche) in 0.5ml glass syringes, and stored at 4^{o}C. Each ampoule contains 3 MIU/0.5ml. A 1:20 dilution of the stock in 1X PBS was prepared and 3.4µl was added to each 1ml of RPMI containing cells to obtain a final concentration of 1000U/ml; This solution was diluted again, 1:100, then 34 µl was added to each 1 ml of RPMI to obtain a final concentration of 100U/ml and 3.4µl was added for a final concentration of 10U/ml.

3. Imatinib

Imatinib is obtained as powder (LC laboratories) and dissolved in dimethyl sulfoxide (DMSO) to obtain a 0.5M stock aliquoted and stored at -20^{o}C; this stock is diluted in 1X PBS to prepare a 25mM solution. For each experiment, a 1:1000 dilution is prepared (25µM) and 40µl were added to each 1 ml of RPMI for a desired final concentration of 1µM.

C. MTT Proliferation Assay and Synergy Analysis

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay is used to measure the in vitro effect of drugs at different concentrations on imatinib – resistant CML cell lines, based on their mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, so any increase or decrease in the number of viable cells is linearly related to

mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals, that are solubilized and measured. Ar230-R and K562-R cells were seeded in triplicates into 96 well plates at a density of $1x10^5$ cells/ml, then treated for 24, 48, and 72 with different drugs concentrations. At each time point, 20µl of MTT dye (Sigma) is added to each well. After 3-4 hours, Formazan crystals are solubilized upon adding 100µl acidified isobutanol solution. The plates were incubated overnight. On the next day, the optical density was measured at wavelength 595 nm using an ELISA microplate.

To evaluate the combination effect of ATO / IFNα on imatinib - resistant CML cells, synergy studies were performed using CompuSyn software, which is based on the CI-isobol method of Chou and Talalay. Using this software, the combination index (CI) is calculated and used to assess synergistic effect (CI < 1), additive effect (CI = 1) or antagonistic effect (CI > 1) of the drug combination.

D. Flow Cytometric Detection of Mitochondrial Membrane Potential

Mitochondrial membrane potential (Δψm) is an important parameter of mitochondrial function and an indicator of cell health. We assessed the mitochondrial membrane potential (ψm) using Rhodamine 123 (Sigma), a cell-permeant, cationic, green-fluorescent dye that is readily sequestered by active mitochondria of living cells. For this assay, cells were seeded and treated for 48 and 72 hours in 6 well plates, washed with PBS, then re-suspended in 500 µl rhodamine 123 washing buffer (130mM sodium chloride, 5mM potassium chloride, 1mM sodium phosphate, 1mM calcium chloride, 1mM MgCl2, 25mM HEPES). Cells are then centrifuged, re-suspended in 500µl rhodamine 123 staining buffer of final concentration of 5µM of rhodamine and

incubated for 30-60 min. at 37°C. Finally the cells are removed from the dye-containing solution and re-suspended in 500µl PBS. Results are instantly read using BD FACSARIA analyzer located in the Physics Department at AUB. Number of events that the flow cytometer acquires is 20 000 cells per sample.

E. Cell Cycle Analysis

Cell cycle analysis was performed using the propidium iodide stain (PI). Cells were seeded at a concentration 1 x 10^5cell/ml and treated with different drug concentrations. Cells were collected after 48 and 72 hours post-treatment, washed with 1X PBS, fixed with ice-cold 70% ethanol, and stored at -20^{o}C for up to ten days. Then, the cells were washed with 1X PBS, and then incubated with RNAse A (Roche) at a final concentration 0.2 mg/ml dissolved in 1X PBS for 45 minutes at 37^{o}C. Subsequently, the cells were stained with PI (0.1 mg/ml) (Sigma). Cell cycle analysis was done using BD FACSARIA analyzer located in the Physics Department at AUB. Number of events that the flow cytometer acquires is 20 000 cells per sample.

F. TUNEL Assay

The TUNEL assay kit (Roche) is used to measure late apoptosis since it detects single and double-DNA strand breaks. The free 3'-OH termini found at the DNA strand breaks can be conjugated to dUTP-fluorescein under the action of the enzyme deoxynucleotidy transferase and fluorescence can be measured through a BD FACSARIA analyzer.

Cells were seeded at a concentration 1x10^5cell/ml and treated with different drug concentrations up to 72 h. Two extra conditions were added for the positive and

negative controls. At the indicated time point, the cells were collected, washed with 1% BSA in 1X PBS, and then fixed in 4% formaldehyde at room temperature for 30 minutes. Subsequently, the cells were washed with 1X PBS and incubated with 100 µL of permeabilization solution (0.1 % Sodium citrate, 0.1% Triton X, and 1X PBS) for 2 minutes on ice. Positive control cells were incubated with 1 µg/ml of DNase for 30 minutes at room temperature, and then washed with 1X PBS. Meanwhile, the cells of the other conditions were washed with 1X PBS. Subsequently, all samples were incubated with TUNEL reagents for 1 h at 37^{o}C in the dark: 50 µl labeling solution for the negative control, and 50 µl of TUNEL reagents mixture for the remaining conditions. The TUNEL reagents mixture is composed of 45 µl labeling solution and 5 µl enzyme solution. Cells were then washed with 1X PBS, re-suspended in 300 µl of 1X PBS, transferred into polystyrene round bottom tubes (Falcon) and fluorescence was measured by BD FACSARIA analyzer located in the Physics Department at AUB. Number of events that the flow cytometer acquires is 20 000 cells per sample.

G. CML Mouse Model

1. Calcium Phosphate Transfection

The ecotropic Platinum E cell lines (plat E) were cultured in complete DMEM containing 1µg/ml of puromycin dihydrochloride and 10µg/ml blasticidin S HCL. Then, the cells were seeded 24 hours before transfection by tripsinization and replating at a density of 5x10^{5} cells/well into 6 well plate in 1ml DMEM without antibiotics. On the day of transfection, 1-2 hours prior transfection, the media was changed. For every two wells, transfection mixture (HEPES-Buffer-Saline + plasmid: Murine IRES GFP bcr-abl T315I + 2M calcium phosphate) was prepared and incubated for 25 minutes at room

temperature, then added to the cells. On the following day, media was changed and the viral supernatant was collected 48-72 hours post transfection.

2. Retroviral Transduction of Bone Marrow Cells

Balb/c mice were injected with 5-Fluorouracil to a final concentration of 200mg/Kg of body weight. Four days later, mice were sacrificed, and bone marrow cells were collected from femurs and tibias. These cells were cultured overnight with complete DMEM containing murine interleukin 3 (IL3) (10ng), murine interleukin 6 (IL6) (10ng), and murine stem cell factor (SCF) (100ng). In the next day and day after, viral supernatant taken from transfected Plat E cells are added to bone marrow cells and spinoculated for 2 hours at 32^{o}C, at 2500 rpm.

Lethally irradiated female Balb/c mice were injected with 2 x10^5 infected bone marrow cells, previously prepared. These mice have been maintained under sterile conditions where they are fed up with sterile food and given acidified water enriched with antibiotics including bactrim.

5 days after the injection, mice were randomly divided into 5 groups: untreated, treated with ATO (2.5g/ kg/day of body weight and injected in the intraperitoneally, treated with IFNα (6x10^5 U/day and injected subcutaneously), treated with imatinib twice a day (50mg/kg/day every morning, and 100mg/kg/day every evening and given gavage), and treated with ATO and IFNα combination. 2 weeks post treatment, half of the mice were sacrificed, and the other half were maintained to check for their survival and physical behavior.

Spleen, liver, lungs, kidney, thymus, and femur were collected from the sacrificed mice and were stained with haematoxylin and Eosin stain for pathological

examination. In addition, serial transplantation assay was performed where 5 million splenocytes were collected from the primary mice and intravenously injected into sub-lethally secondary mice that were left untreated and maintained under daily observation.

Chapter III

Results

A. In Vitro Results

1. Effect of ATO/IFNα treatment on the growth of imatinib-resistant CML cell lines

We treated two imatinib-resistant human CML cell lines, Ar230-R and K562-R for 24, 48 and 72 hours, with different concentrations of ATO and/or IFNα. Achievable pharmacological concentrations of ATO (1 μM or 2 μM) and IFNα (100 IU/ml or 1000 IU/ml) were used in these experiments.

To study the effect of ATO and/or IFNα treatment on cell growth inhibition of Ar230-R and K562-R CML cells, MTT cell proliferation assay was used. Notably, Imatinib had minimal effect on cell proliferation in Ar230-R and K562-R cells after 72 hours, indicating resistance to imatinib treatment (Figure 20 *A* and *B*).

Our results show minimal growth inhibition in CML cells treated with IFNα alone, and relatively increased inhibition in cells treated with ATO alone, however growth inhibition becomes higher in cells treated with ATO/IFNα combination. In both cell lines, IFNα 100 or IFNα 1000 alone treatment had no effect on cell proliferation at 24 hours, and had minimal effect after 72 hours post treatment. In addition, cells treated with ATO alone show time-dependent inhibition that reaches around 15%-30% after 48 hours of treatment and increases up to 35%-45% 72 hours post treatment (Figure 21). Interestingly, in Ar230-R cells, the effect of the combination on growth inhibition shows minimal effect after 24 hours post-treatment; that became clear after 48 hours, where synergy effect was seen in cells treated with the different concentrations. At 72 hours post treatment, we observed a pronounced inhibition of proliferation in Ar230-R cells treated with the combination compared to each drug alone. In addition, synergy

effect between ATO and IFNα was clear in cells treated with all concentrations of the combination except for cells treated with ATO1/IFNα 1000 where similar effect was seen in cells treated with ATO alone (Figure 21 *A*, *B* and *E*).

On the other hand, K562-R cells treated with ATO/IFNα combination show time-dependent growth inhibition, reaching 15% - 30% after 48 hours, and increases to reach 35% - 50% after 72 hours. Synergy effect was clear after 48 hours in cells treated with ATO1/IFNα 100 and ATO1/IFNα 1000. However, synergy is preserved after 72 hours in cells treated with all concentrations of the combination (Figure 21 *C*, *D* and *F*). Indeed, in both cell lines, our observations show a relatively similar sensitivity to ATO/IFNα treatment evident by the growth inhibition of both cell lines reaching around 50% after 3 days of treatment. This growth inhibition was both time and dose dependent (Figure 21).

Synergy analysis was performed using the method of Cho and Talalay where it showed a synergy effect between ATO and IFNα confirmed by a combination index below 1 (Figure 21 *E* and *F*).

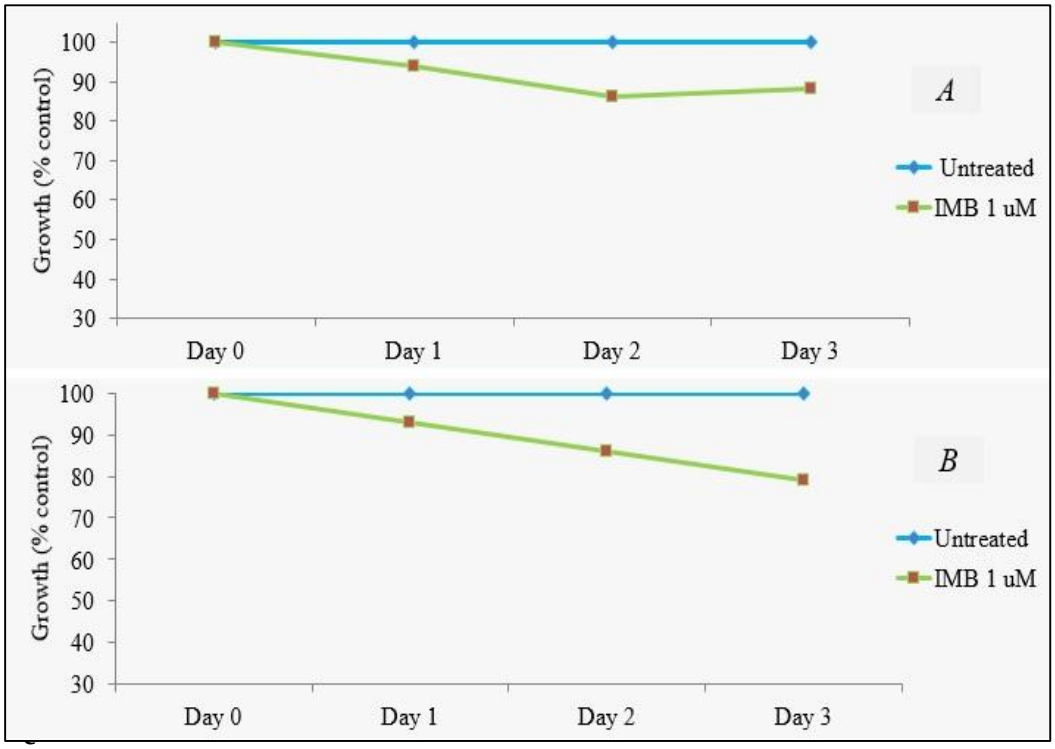


Figure 20 (A and *B*): Imatinib has minimal effect on the proliferation of Ar230-R and K562-R cells.

Imatinib has minimal effect on Ar230-R and K562-R cells. Ar230-R (*A*) and K562-R (*B*) cells were seeded at a concentration of $1x10^5$ cells/ml and treated with imatinib 1 µM up to three days, as indicated. Cell growth was assayed in triplicate using MTT cell proliferation assay, and pooled data are represented. The results are expressed as percent of untreated sample. Results are the average of three independent experiments. IMB denotes imatinib.

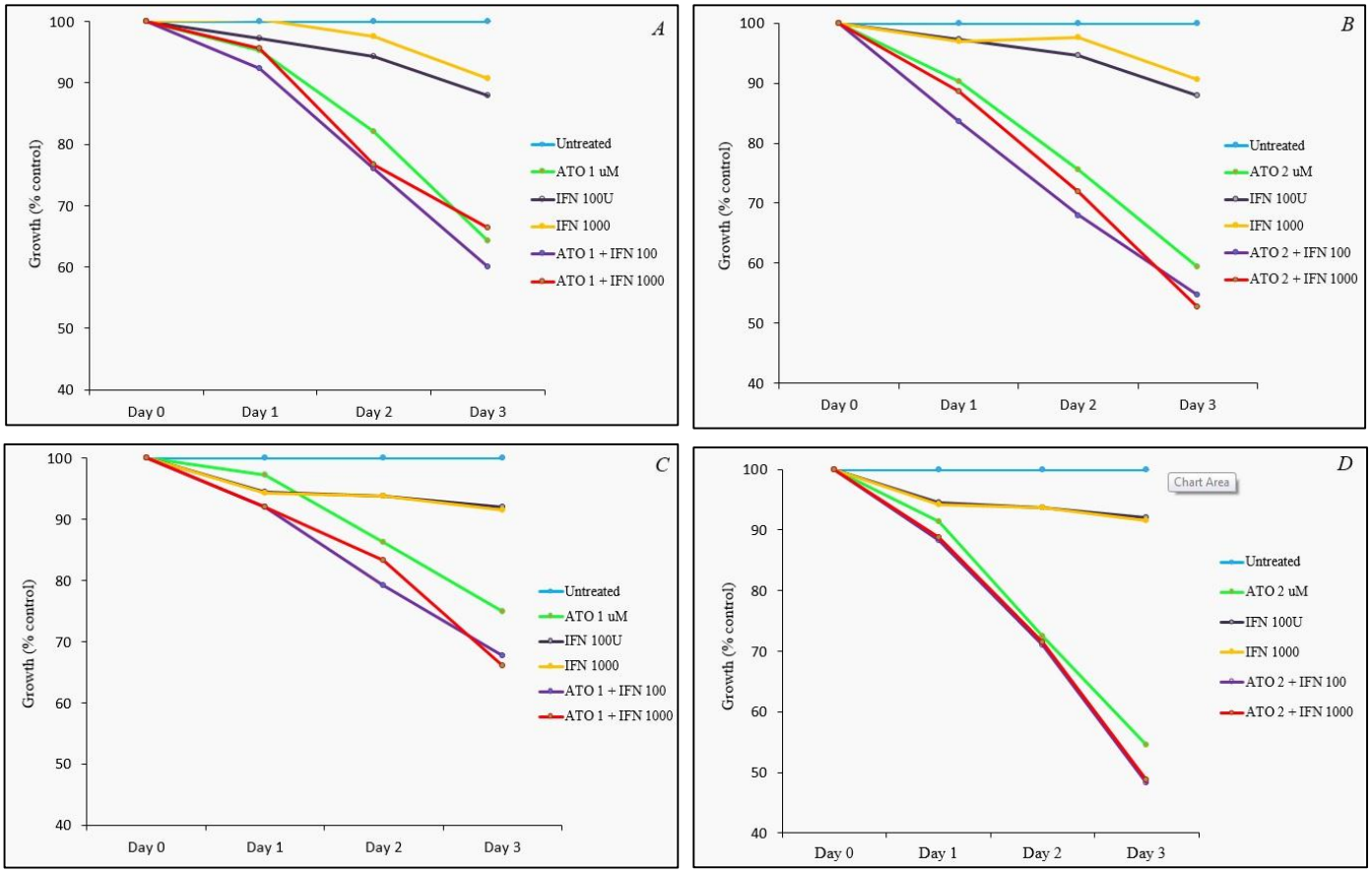


Figure 21 (*A*, *B*, *C* and *D*): Ar230-R (*A* and *B*) and K562-R (*C* and *D*) cells are sensitive to ATO/IFNα treatment.

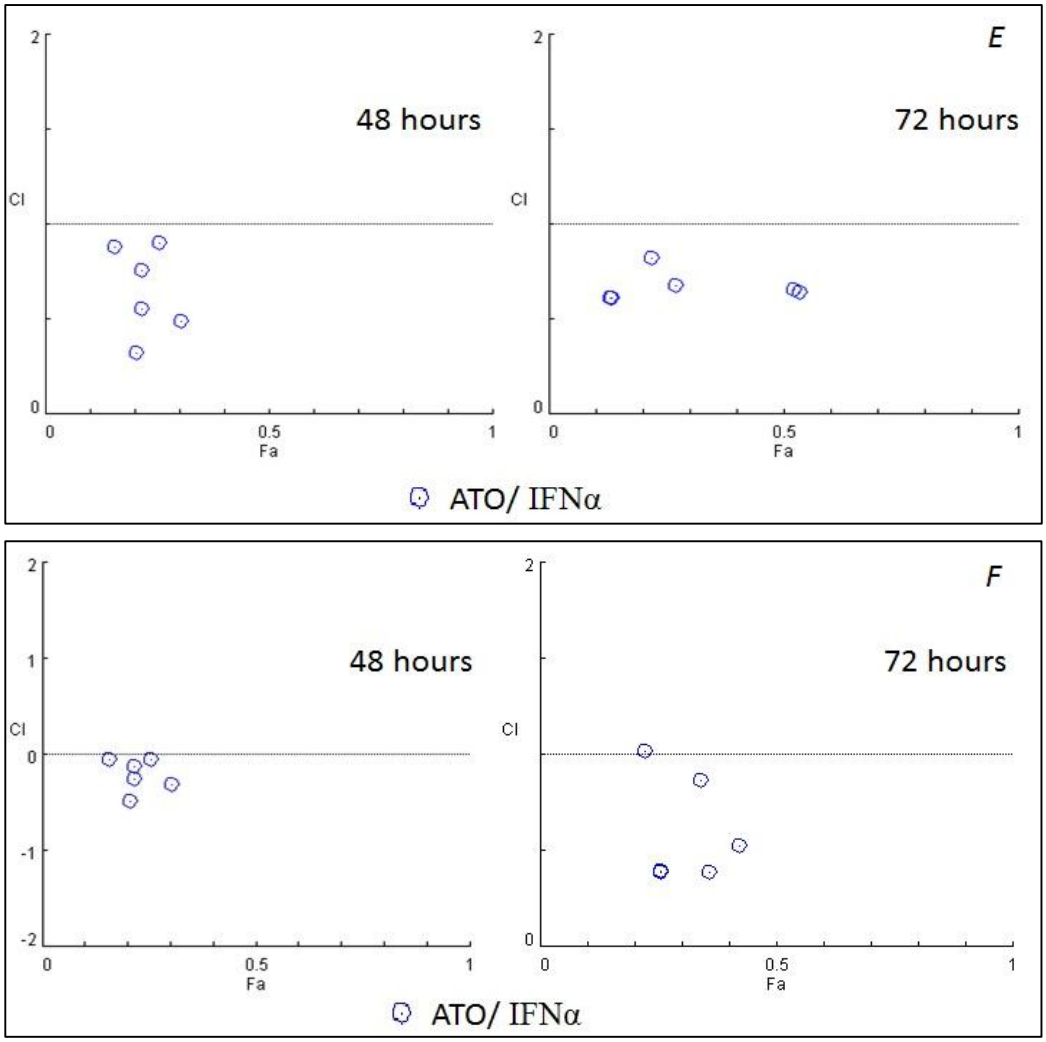


Figure 21 (*E* and *F*): ATO/IFNα show a synergistic effect evident by combination index below 1 in Ar230-R cells (*E*) K562-R cells (*F*). CI<1, CI=1, CI>1 represent synergistic, additive, or antagonistic effect of both drugs, respectively.

ATO and IFNα exhibit an anti-proliferative synergistic effect in both imatinib resistant CML cell lines. Ar230-R (A,B) and K562-R (C,D) cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) up to three days, as indicated. Cell growth was assayed in triplicate using MTT cell proliferation assay, and pooled data are represented. The results are expressed as percent of untreated sample. Results are the average of three independent experiments. Synergy analysis based on the CI-isobol method of Chou-Talalay showed a synergy between ATO and IFNα evident by combination index below 1 (E.F). ATO denotes arsenic trioxide and IFNα denotes interferon alpha.

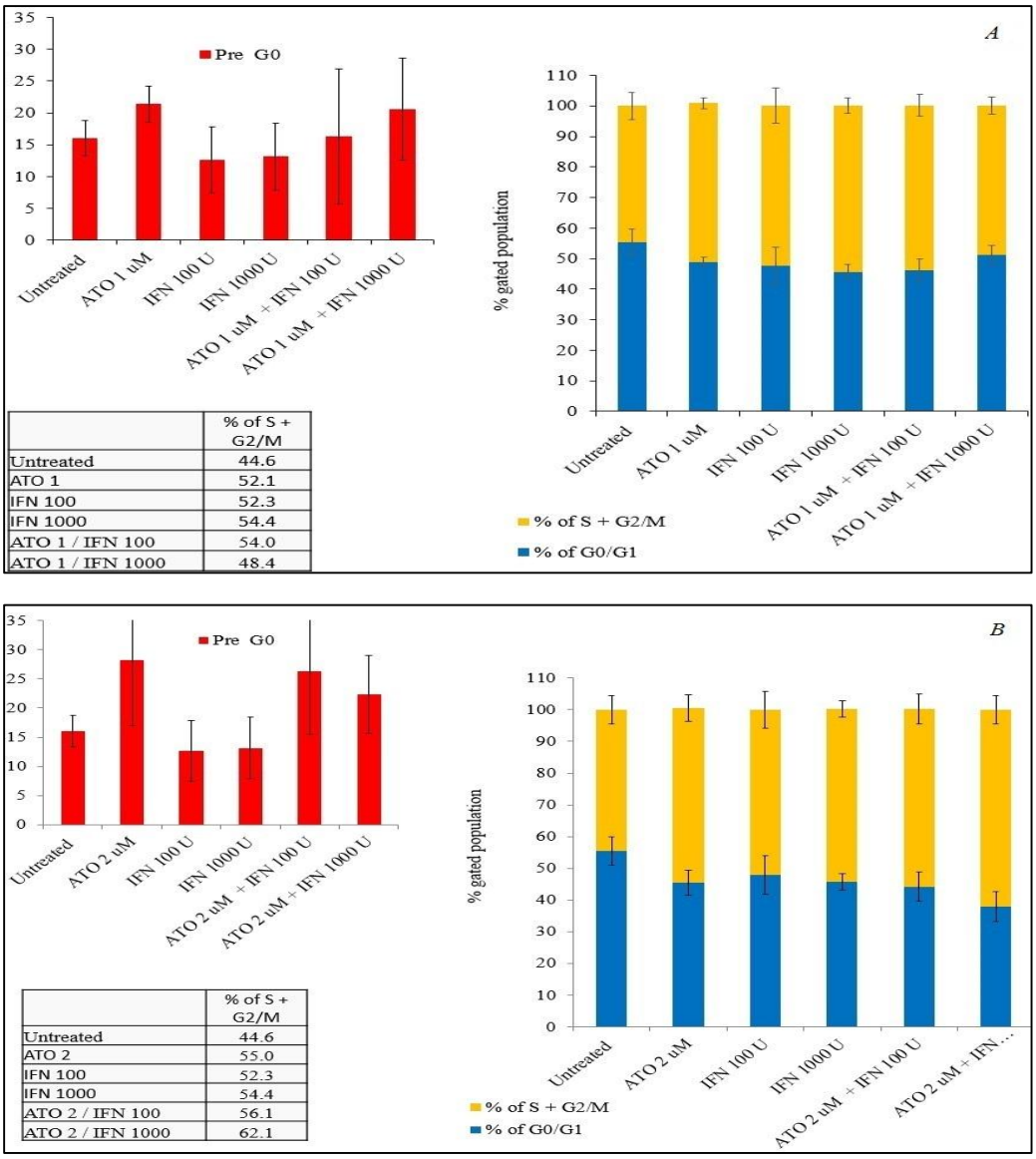
2. Effect of ATO/IFNα treatment on cell cycle of imatinib-resistant CML cell lines

In order to investigate the mechanism of cell death induced by ATO/IFNα treatment, we performed cell cycle analysis in the two imatinib-resistant CML cell lines 72 hours post treatment using propidium iodide staining.

Ar230-R (Figure 22 *A* and *B*) and K562-R cells (Figure 22 *C* and *D*) were treated with ATO (1 μM or 2 μM) and/or IFNα (100 IU/ml or 1000 IU/ml) for 72 hours, then stained with PI (20 μg/ml), and cell cycle analysis was performed using BD FACSARIA flow cytometer.

Our results show that treatment with ATO/IFNα results in the induction of cell cycle arrest of imatinib-resistant CML cells in S+G2/M phase of the cell cycle 72 hours post treatment. This cell cycle arrest appears to be pronounced with the combination specifically in cells treated with ATO 2 μM reaching approximately 60% compared to 45% S+G2/M cell cycle arrest in untreated imatinib-resistant CML cells (Figure 22 and 23).

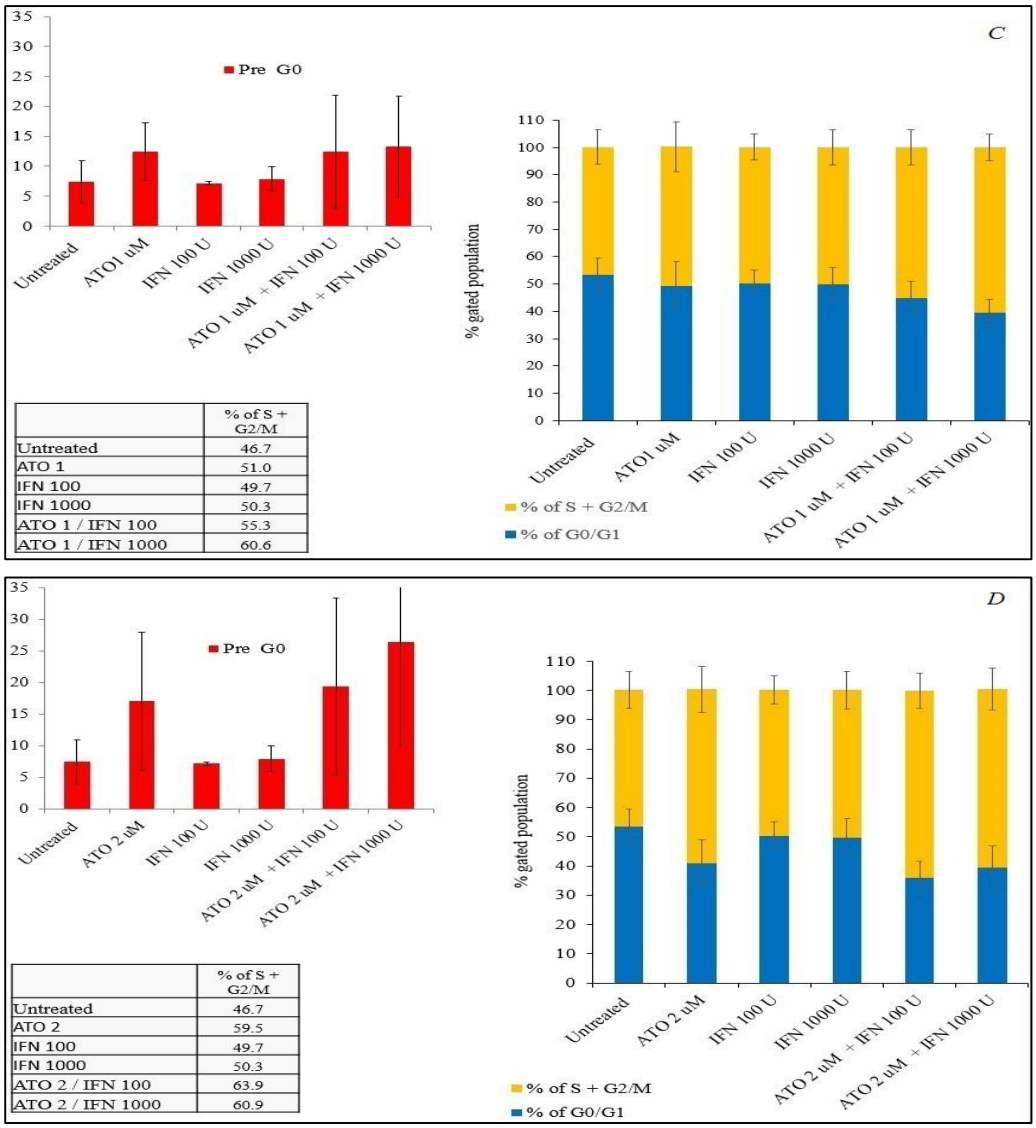
In addition, our results show an increase in the percentage Pre G0 phase of the cell cycle in cells treated with ATO or ATO/IFNα combination compared to untreated cells. This reflects that ATO alone or in combination with IFNα is probably inducing cell apoptosis that is clear after 72 hours of treatment, however, this needs to be confirmed using other apoptotic assays (Figure 22 and 23).



	% of S + G2/M
Untreated	44.6
ATO 1	52.1
IFN 100	52.3
IFN 1000	54.4
ATO 1 / IFN 100	54.0
ATO 1 / IFN 1000	48.4

	% of S + G2/M
Untreated	44.6
ATO 2	55.0
IFN 100	52.3
IFN 1000	54.4
ATO 2 / IFN 100	56.1
ATO 2 / IFN 1000	62.1

Figure 22 (*A* and *B*): ATO/IFNα treatment of Ar230-R cells induces S+G2/M cell cycle arrest.



	% of S + G2/M
Untreated	46.7
ATO 1	51.0
IFN 100	49.7
IFN 1000	50.3
ATO 1 / IFN 100	55.3
ATO 1 / IFN 1000	60.6

	% of S + G2/M
Untreated	46.7
ATO 2	59.5
IFN 100	49.7
IFN 1000	50.3
ATO 2 / IFN 100	63.9
ATO 2 / IFN 1000	60.9

Figure 22 (*C* and *D*): ATO/IFNα treatment of K562-R cells induces S+G2/M cell cycle arrest.

ATO/IFNα treatment of imatinib-resistant CML cells induces S+G2/M cell cycle arrest. Ar230-R and K562-R cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) for 72hours, then stained with PI (20 µg/ml), and fluorescence was instantly measured by BD FACSARIA analyzer. Pre G0 represents the presumably apoptotic cells. The sum of S+G2/M, which represents the cycling cells, is a percentage of non-apoptotic cells. Graphs are the average of 3 independent experiments. ATO denotes arsenic trioxide and IFNα denotes interferon alpha. Error bars show Standard Deviations.

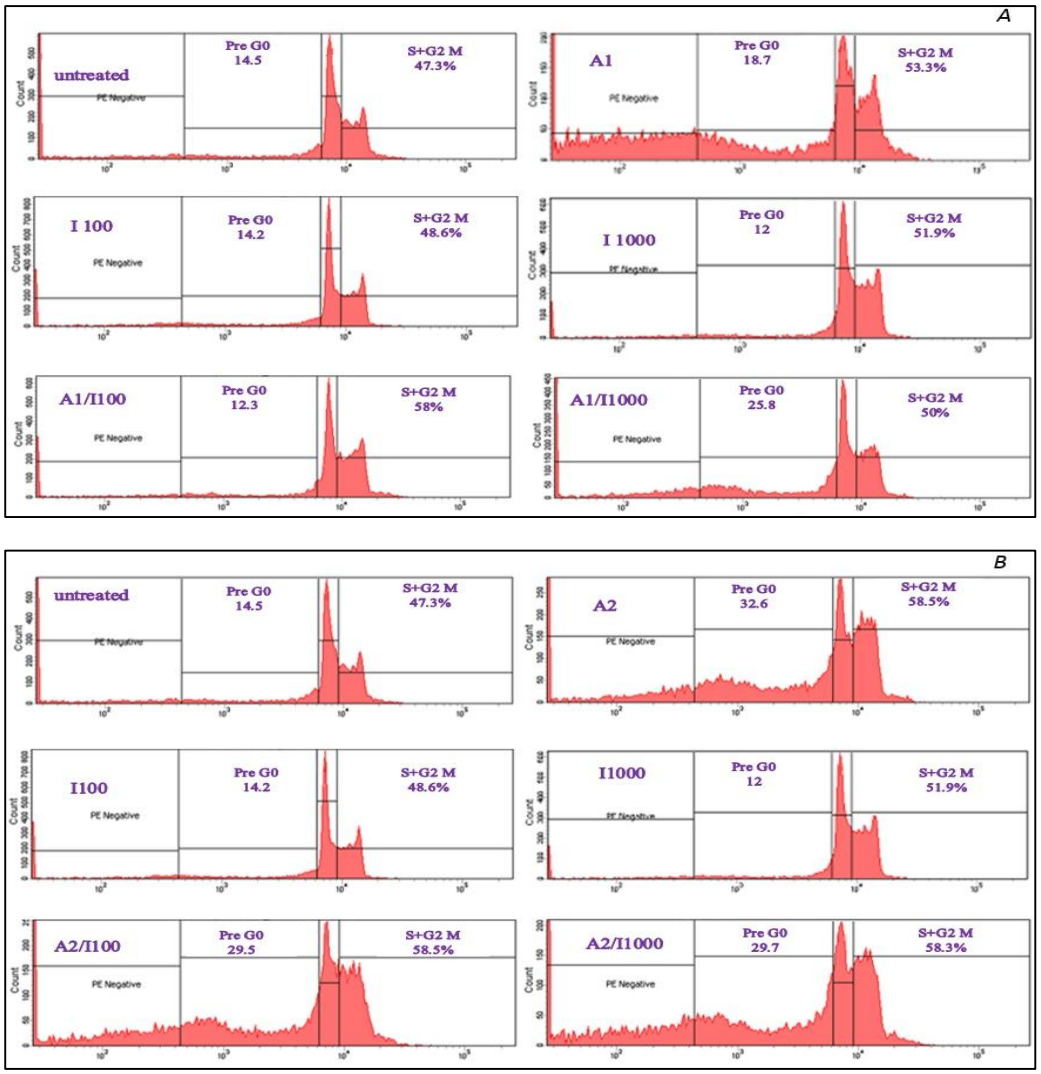


Figure 23 (*A* and *B*): Cell Cycle analysis of Ar230-R cell lines using Propidium Iodide staining.

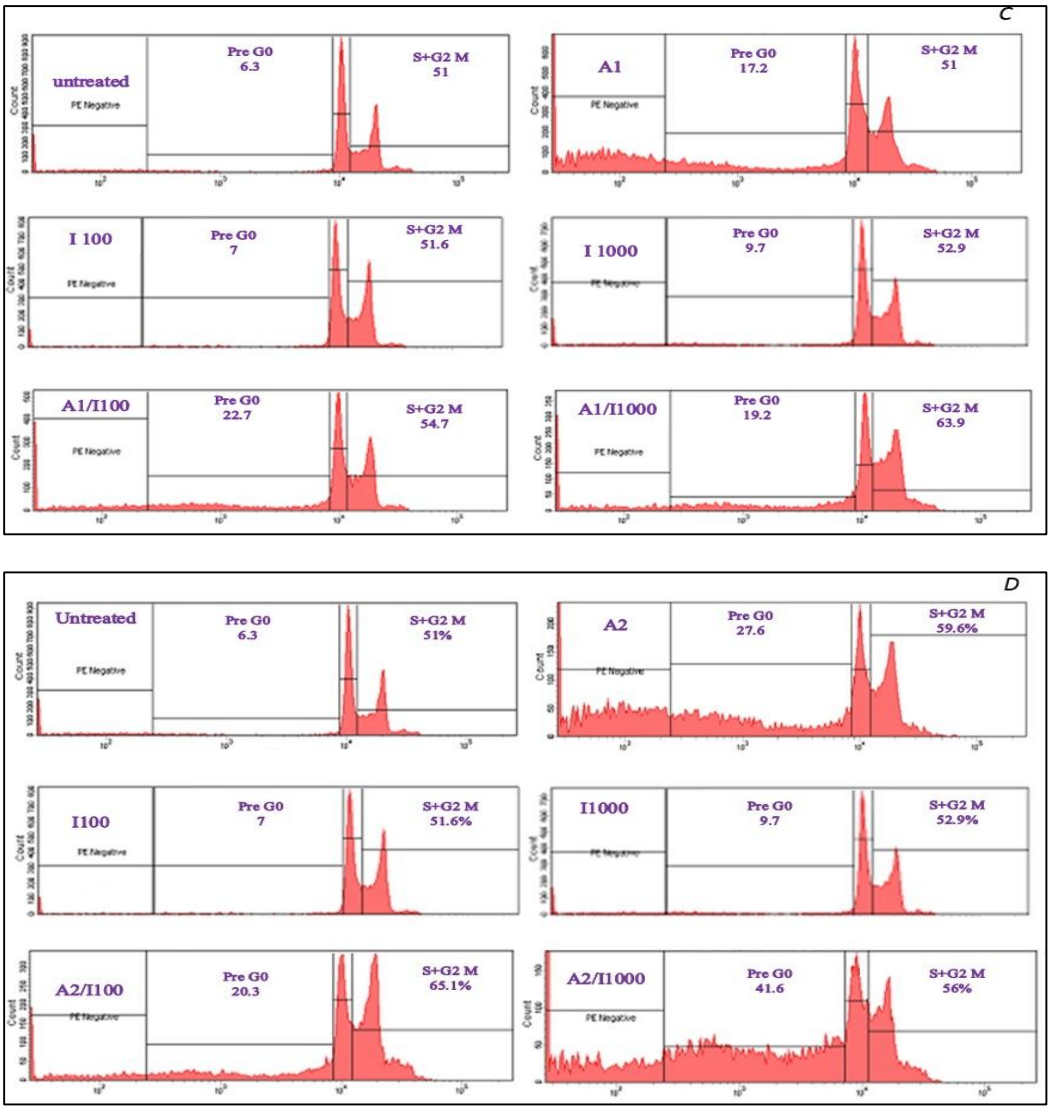


Figure 23 (*C* and *D*): Cell Cycle analysis of K562-R cell lines using Propidium Iodide staining.

ATO/IFNα treatment of imatinib-resistant CML cells induces S+G2/M cell cycle arrest. Ar230-R and K562-R cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) for 72hours, then stained with PI (20 µg/ml), and fluorescence was instantly measured by BD FACSARIA analyzer. Pre G0 represents the presumably apoptotic cells. The sum of S+G2/M which represents the cycling cells are a percentage of non-apoptotic cells. Histograms represent results of one experiment chosen to represent the results of 3 independent experiments. "A" denotes arsenic trioxide and "I" denotes interferon alpha.

3. Effect of ATO/IFNα treatment on the induction of apoptosis in CML K562-R cells

To confirm the induction of apoptosis shown by the increase in Pre G0 cells in ATO/IFNα treated imatinib-resistant CML cells, we performed the TUNEL assay that detects DNA cleavage. K562-R cells were treated with ATO (1 μM or 2 μM) and/or IFNα (1000 IU/ml) for 72 hours.

Interestingly, compared to untreated cells, the percentage of TUNEL-positive cells, i.e. apoptotic cells, increased from 6.3% to 33.4% in cells treated with the combination ATO1/IFNα1000 and increased more in cells treated with an increased concentration of ATO (2 μM) to reach approximately 50%. IFNα alone had the least effect on DNA fragmentation, however ATO shows a dose-dependent effect on the induction of apoptosis. As expected, at 72 hours post treatment, cells treated with ATO 2 μM had the same effect on DNA fragmentation as those treated with the combination ATO 2 μM and IFNα 1000 IU/ml (Figure 24).

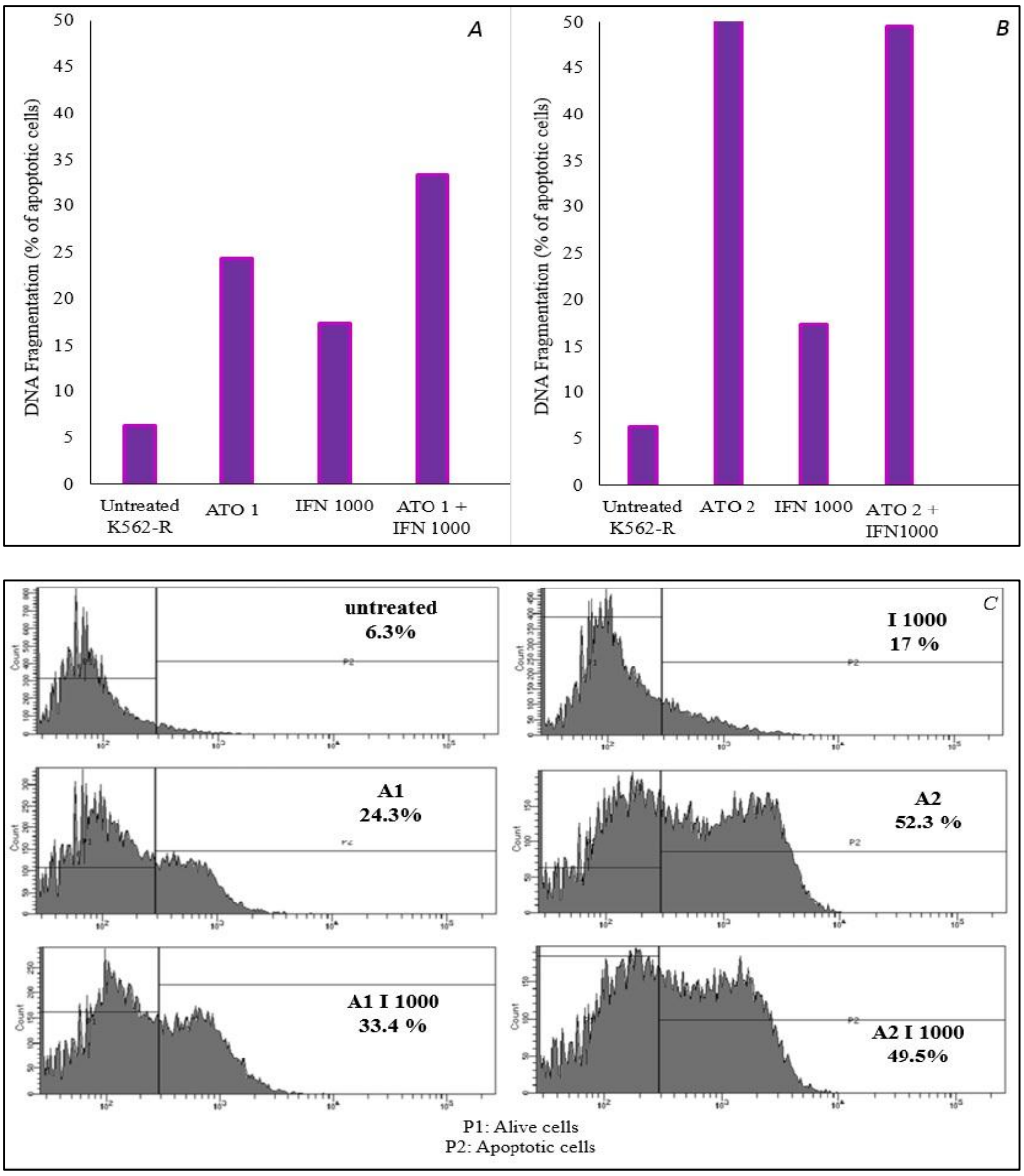


Figure 24 (*A*, *B* and *C*): ATO/IFNα treatment induces apoptosis in K562-R cells.

ATO/IFNα treatment induces apoptosis in imatinib-resistant CML cells. K562-R cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (1000 IU/ml) for 72 hours. TUNEL assay was performed, and fluorescence was instantly measured by BD FACSARIA analyzer. These are the results of one experiment. Histograms represent the results of the experiment done on K562-R cells using FACSARIA analyzer (C). ATO and A denote arsenic trioxide and IFNα and I denote interferon alpha.

4. Effect of ATO/IFNα treatment on mitochondrial membrane potential of imatinib-resistant CML cell lines

To investigate the effect of ATO/IFNα treatment on the mitochondrial membrane potential, we performed Rhodamine assay that detects the dissipation of the mitochondrial membrane potential, an early apoptotic marker and evident by the decrease in Rhodamine-123 fluorescence.

For that, Ar230-R (Figure 25 *A, B, C* and *D*) and K562-R (Figure 25 *E, F, G* and *H*) cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) up to three days, then stained with Rhodamine-123 (5 µM), and instantly read using BD FACSARIA analyzer.
IFNα treatment is seen to have minimal effect on mitochondrial membrane potential compared to ATO treatment that shows a higher effect. Additionally, the loss of mitochondrial potential is seen to be ATO dose-dependent and simultaneously increases with the increase in the concentration of ATO from 1 µM or 2 µM. Moreover, our results show that in both cell lines, the loss of mitochondrial potential was highest in cells treated with the combination compared to those treated with either one of the drugs. In addition, ATO/IFNα- induced apoptosis in imatinib-resistant CML cell lines was associated with dose and time-dependent dissipation of the mitochondrial membrane potential, where results show increased loss at 72 hours post treatment compared to 48 hours (Figure 25 and 26).

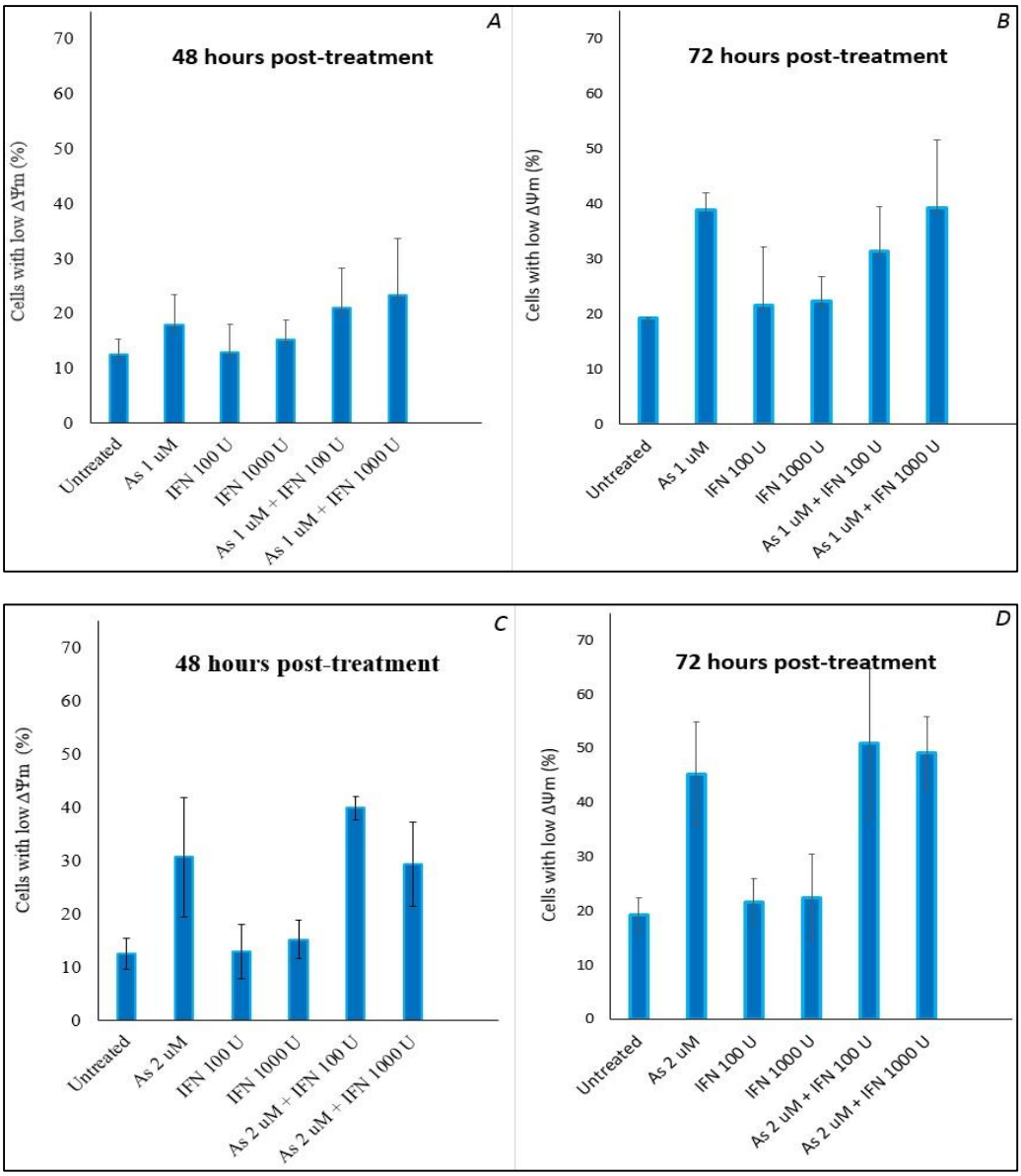


Figure 25 (*A*, *B*, *C* and *D*): ATO/IFNα treatment of Ar230-R cells results in loss of mitochondrial membrane potential.

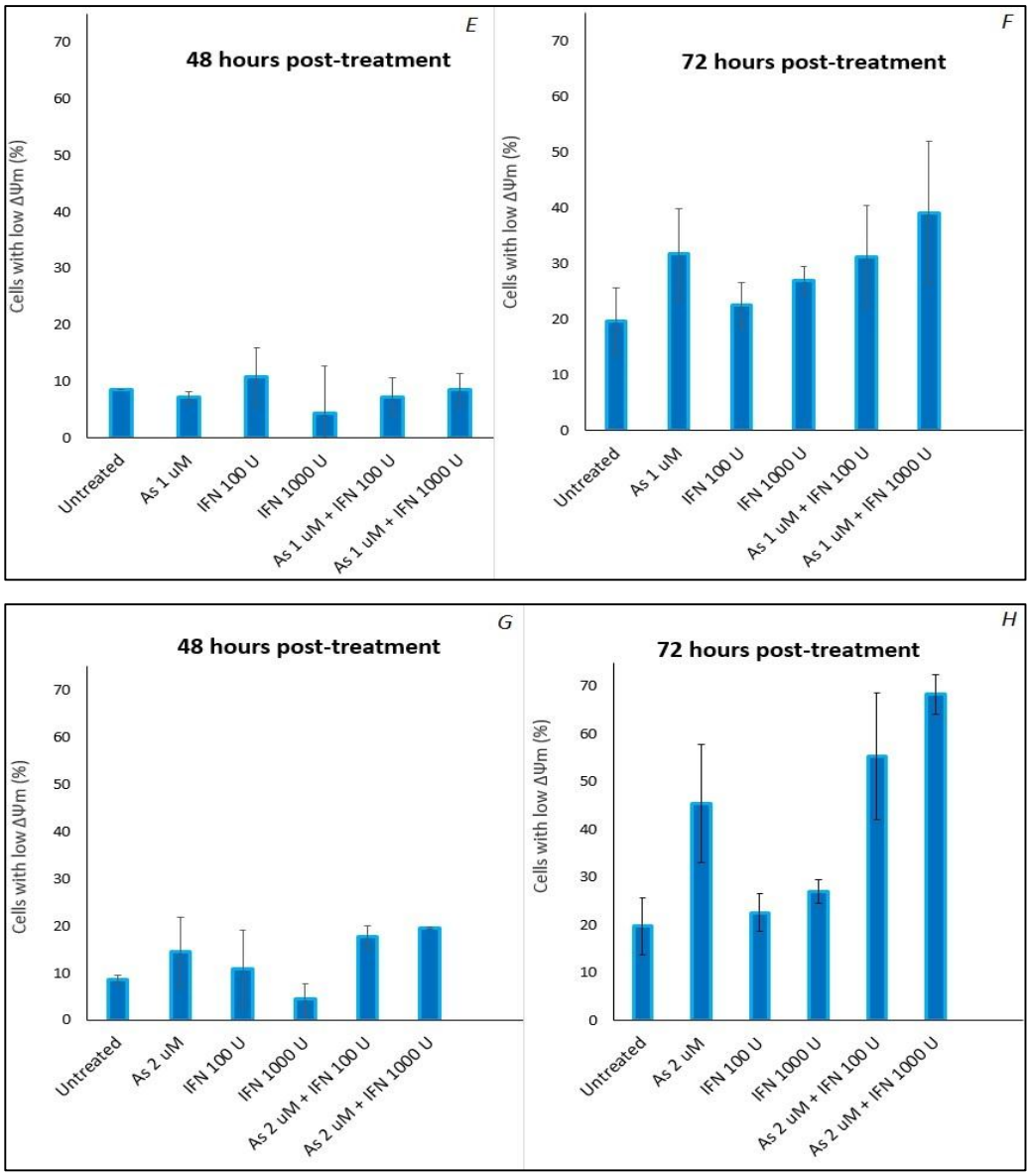


Figure 25 (*E*, *F*, *G* and *H*): ATO/IFNα treatment of K562-R cells results in loss of mitochondrial membrane potential.

ATO/IFNα treatment of imatinib-resistant CML cells, results in loss of mitochondrial membrane potential. Ar230-R and K562-R cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) up to three days, then stained with Rhodamine-123 (5 µM), and instantly read using BD FACSARIA analyzer. Graphs are the average of 3 independent experiments. ATO denotes arsenic trioxide and IFNα denotes interferon alpha. Error bars show Standard Deviations.

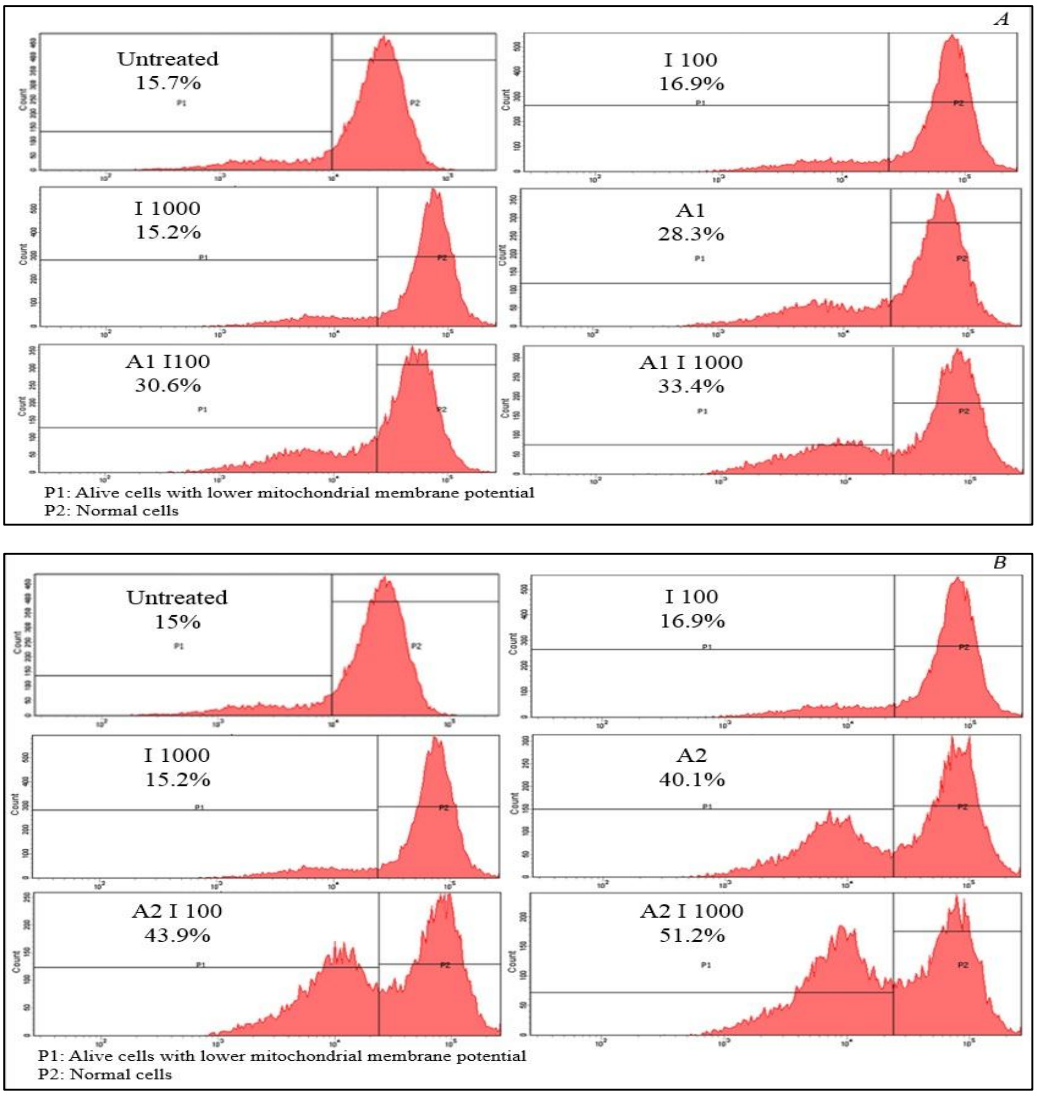


Figure 26 (*A* and *B*): Mitochondrial membrane potential analysis of Ar230-R cell lines using Rhodamine-123 Staining.

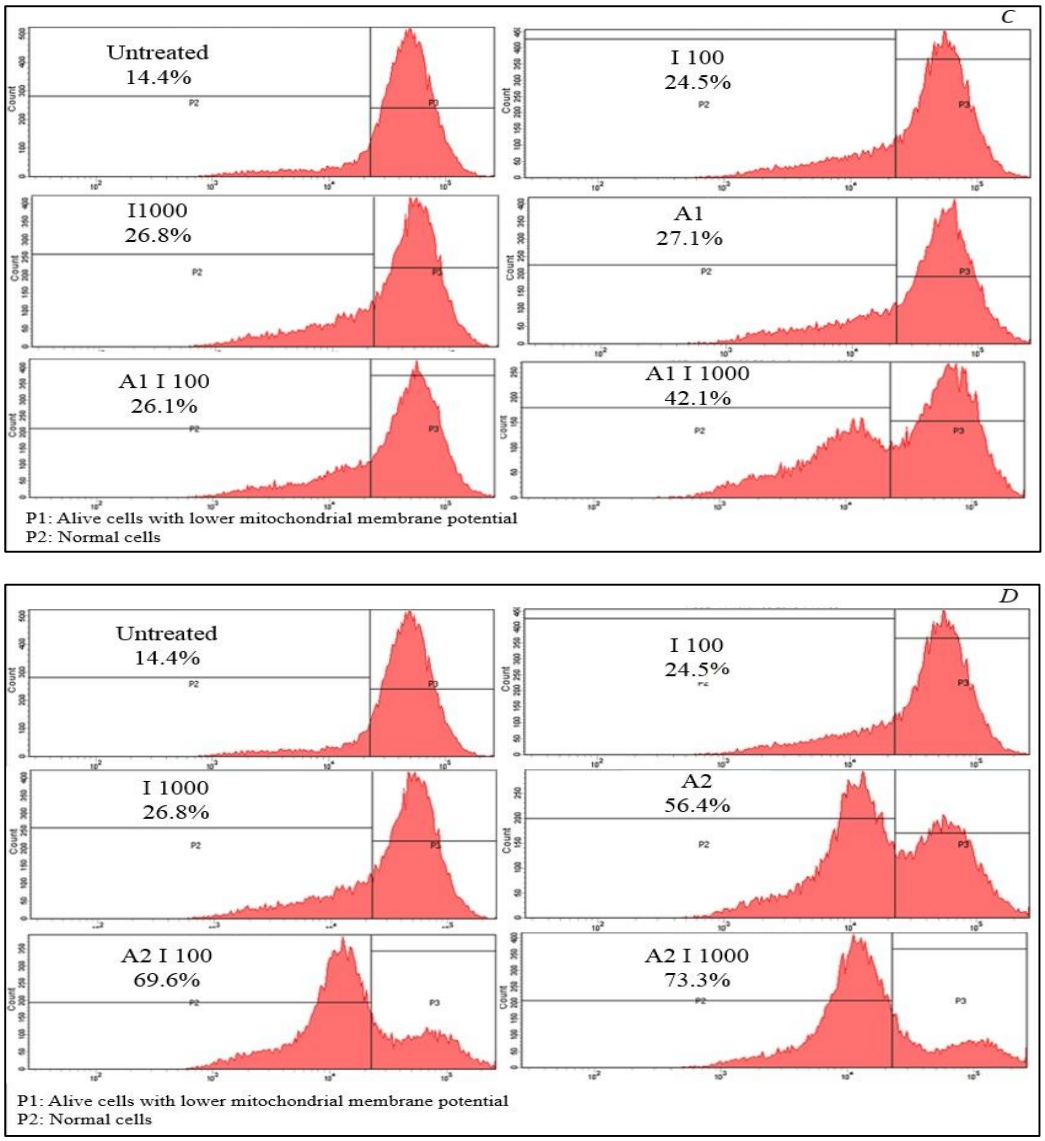


Figure 26 (*C* and *D*): Mitochondrial membrane potential analysis of K562-R cell lines using Rhodamine-123 Staining.

ATO/IFNα treatment of imatinib-resistant CML cells, results in loss of mitochondrial membrane potential. Ar230-R (*A* and *B*) and K562-R (*C* and *D*) cells were seeded at a concentration of $1x10^5$ cells/ml and treated with ATO (1 µM or 2 µM) and/or IFNα (100 IU/ml or 1000 IU/ml) up to 3 days, then stained with Rhodamine-123 (5 µM), and instantly read using BD FACSARIA analyzer. Histograms represent results of one experiment chosen to represent the results of 3 independent experiments. "A" denotes arsenic trioxide and "I" denotes interferon alpha.

B. Invivo Results

In vitro results show promising preliminary data of the effect of ATO/ IFNα combination of imatinib-resistant cells. For that we proceeded to investigate the influence of ATO/ IFNα on T315I-CML mouse model that we reproduced in our lab.

1. Description of the BCR-ABL T315I mouse model

Bcr-abl T315I-CML mouse model is a new CML model that was used only in few papers (A, B). Briefly, Plat E cells were transfected with murine IRES GFP bcr-abl T315I plasmid, then viral supernatant was collected. The efficiency of transfection was analyzed by reading % of GFP-positive cells in PLAT E cells using flow cytometry that revealed average of 15%-50% efficiency. Viral supernatant was added to bone marrow cells taken from 5-fluorouacil-treated Balb/c mice. 2 day later, bone marrow cells were transduced, collected and $2x10^5$ cells were injected intravenously into lethally irradiated primary recipients. The efficiency of transduction was analyzed by reading % of GFP-positive cells in a sample of infected bone marrow cells using flow cytometry that revealed an average of 30% to 70% (Figure 27). Mice were monitored for the survival. Leukemic mice started to die at 2-3 weeks post injection (Figure 30).

Importantly, when morbid mice were sacrificed, we examined some unique observation in the leukemic mice that we did not experience with primary wild-type CML mice. We observed in primary mice splenomegaly, hepatomegaly, infiltration of the spleen and liver, and thymus and heart enlargement. Interestingly, CBC count test on blood samples taken from untreated and normal Balb-c mice through eye bleeding technique, show a huge increase in the number of white blood cells, neutrophils and lymphocytes by 10X, 1.7X, and 15X increase, respectively relative to the CBC count of

normal Balb-c blood sample (29 A). This increase in the lymphocytes was not seen in primary CML wild-type mice. Moreover, the spleen and liver weight largely increased in untreated T315I mice to reach 678.1 mg and 2.47 g, respectively, compared to normal Balb-c mice that show spleen weight of only 85.3 mg and liver weight of 1,47g (29 C and D).

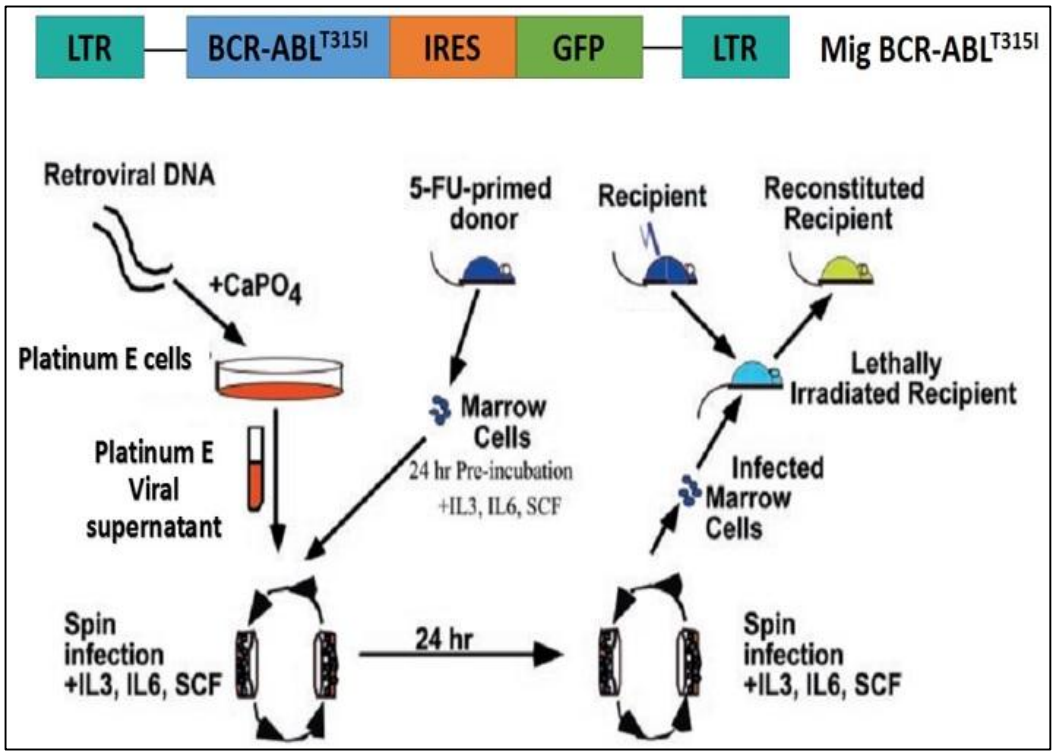


Figure 27: Schematic representation of BCR-ABL T315I CML Mouse Model.

Plat E cells were transfected with murine IRES GFP bcr-abl T315I plasmid, then viral supernatant was collected. Viral supernatant was added to bone marrow cells taken from 5-fluorouacil-treated Balb/c mice. 2 day later, transduced bone marrow cells were collected and $2x10^5$ were injected intravenously into lethally irradiated primary recipients. Primary mice were kept under sterile conditions where they are fed up with sterile food and given acidified water enriched with antibiotics. Leukemic mice were monitored for their survival.

2. Effect of ATO/IFNα treatment on the survival of primary BCR-ABL T315I CML mice

To study the effect of treatment on leukemic mice survival, T315I mice were randomly distributed, 5- days post injection, between five groups: untreated, treated intraperitoneally with ATO (2.5g/ kg/day of body weight), treated subcutaneously with IFNα ($6x10^5$ IU/day), treated with ATO and IFNα combination, or treated by gavage with imatinib twice a day (50mg/kg/day every morning, and 100mg/kg/day every evening).

Two experiments were done as described above, however primary untreated mice started to die after two weeks post injection. For that, in the third experiment, we diluted the viral supernatant. This change in the concentration of the viral supernatant caused the prolonged survival of untreated mice reaching to 3 weeks post injection in mice of the third experiment.

2 weeks post treatment, half of the mice were sacrificed, and the other half were maintained to check for their survival and physical behavior (Figure 28). Interestingly, the number of white blood cells and lymphocytes decreased largely in blood samples taken from all treated mice. On the other hand, the number of neutrophils was the approximately the same in blood samples taken from mice treated with imatinib or ATO alone compared to the sample taken from untreated mice; however, the number increases in samples taken from mice treated with IFNα and doubles in samples taken from mice treated with the combination (Figure 29 *A*). Moreover, flow cytometry analysis revealed a decrease in the percentage of GFP^+ blood cells taken from mice treated with the combination, however, to a lesser extent in mice treated with either drugs or imatinib (Figure 29 *B*). This event possibly reflects the effect of the

combination on the eradication of leukemic progenitor cells. In all treated mice, the weight of the spleen and liver did not show any variations compared to untreated mice (Figure 29 *C* and *D*).

Moreover, based on the Kaplan-Meier plot, most untreated mice and mice treated with imatinib died after 3 weeks of treatment, as expected. The median for survival ranged from 21-30 days in untreated mice and 15-34 days in imatinib-treated mice. Treatment of mice with IFNα or ATO alone had no effect on leukemic mice survival. However, ATO/IFNα combination prolonged the survival of mice. Indeed the mice treated with ATO/IFNα combination died few weeks later to reach an upper median of 46 days, in time some mice survived till 60-75 days. This reflects the resistance of T315I-CML mice model to imatinib treatment, and its sensitivity to ATO/IFNα combination (Figure 30).

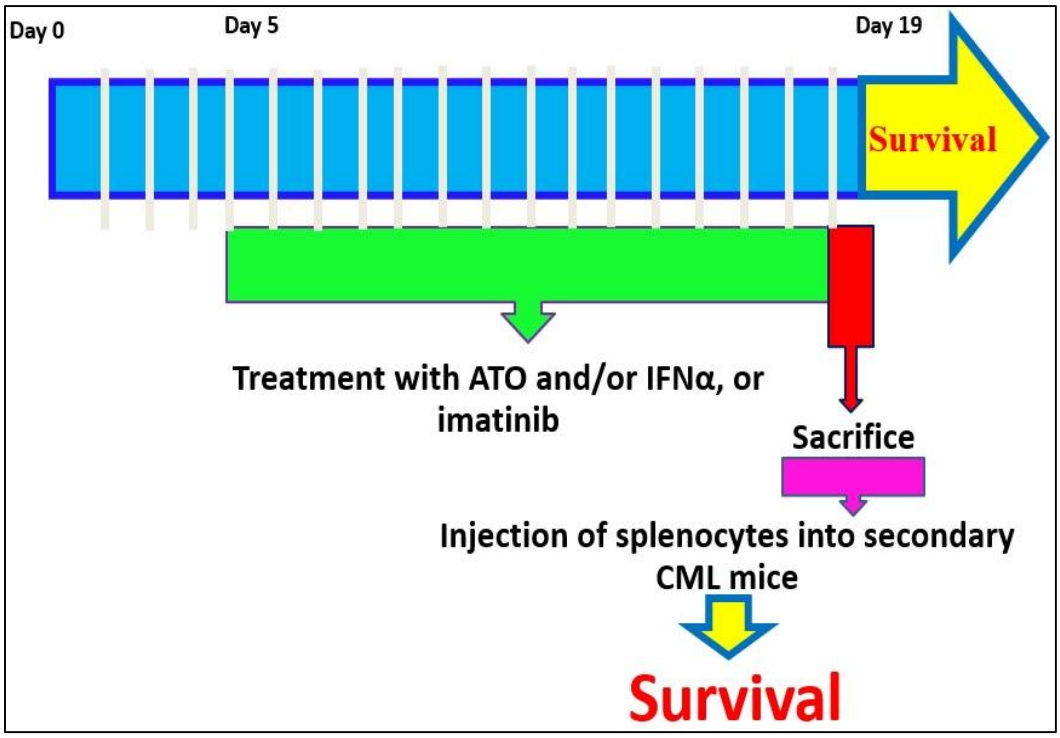
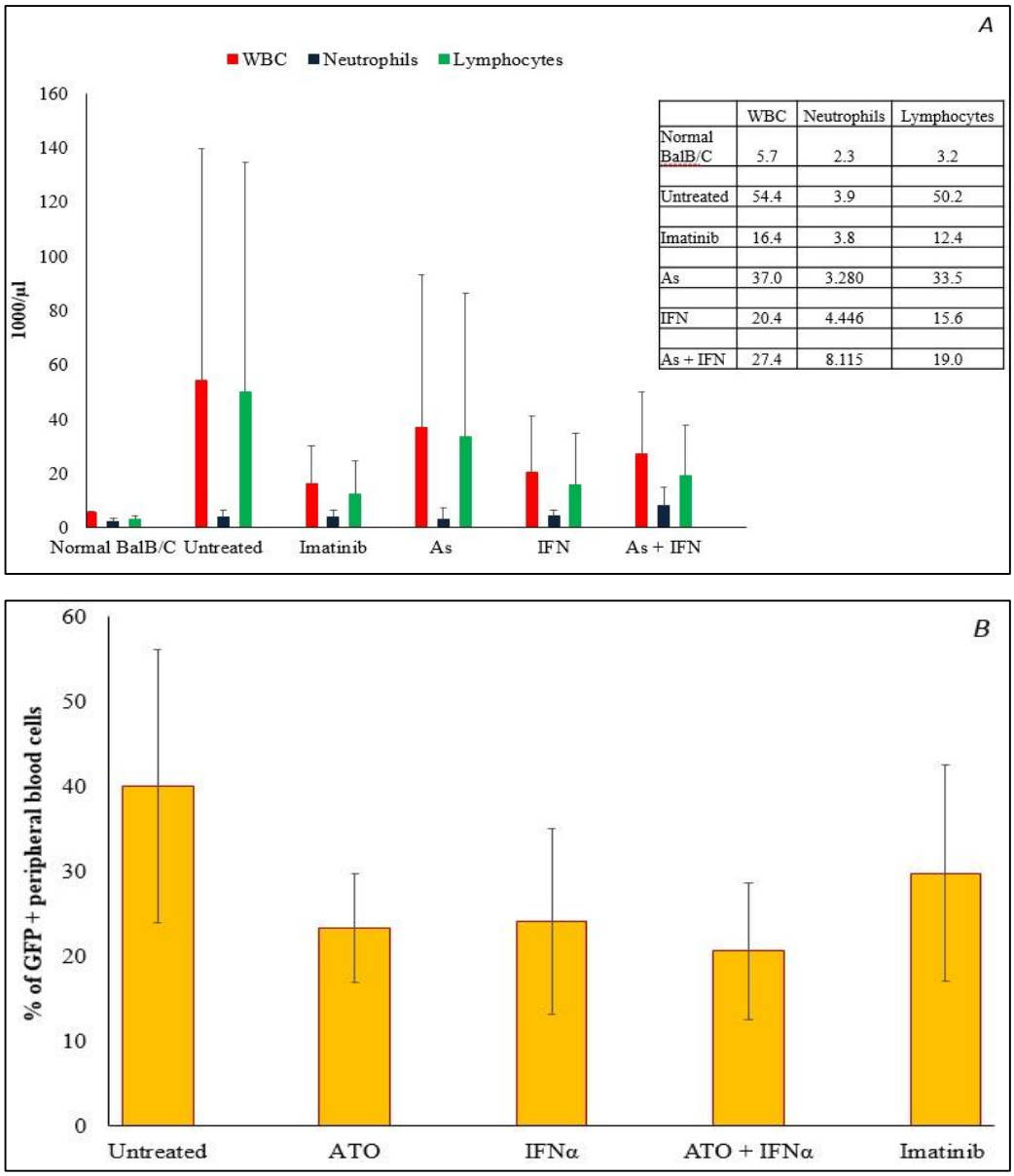


Figure 28: Experimental Plan of in vivo experiments.

5 days post transplantation of bone marrow transduced cells, leukemic mice were left untreated or treated with ATO and/or IFNα or imatinib for 2 weeks. Some of the mice were sacrificed at the end of the treatment while the rest of the animals were monitored for survival. Serial transplantation of splenocytes (5×10^6 cells) from primary T315I-CML mice left untreated or treated for 2 weeks into syngeneic secondary recipients, which were not subsequently treated, was performed.



	WBC	Neutrophils	Lymphocytes
Normal BalB/C	5.7	2.3	3.2
Untreated	54.4	3.9	50.2
Imatinib	16.4	3.8	12.4
As	37.0	3.280	33.5
IFN	20.4	4.446	15.6
As + IFN	27.4	8.115	19.0

Figure 29 (*A* and *B*): Effect of ATO/IFNα treatment on blood cells and the percentage of GFP$^+$ in peripheral blood.

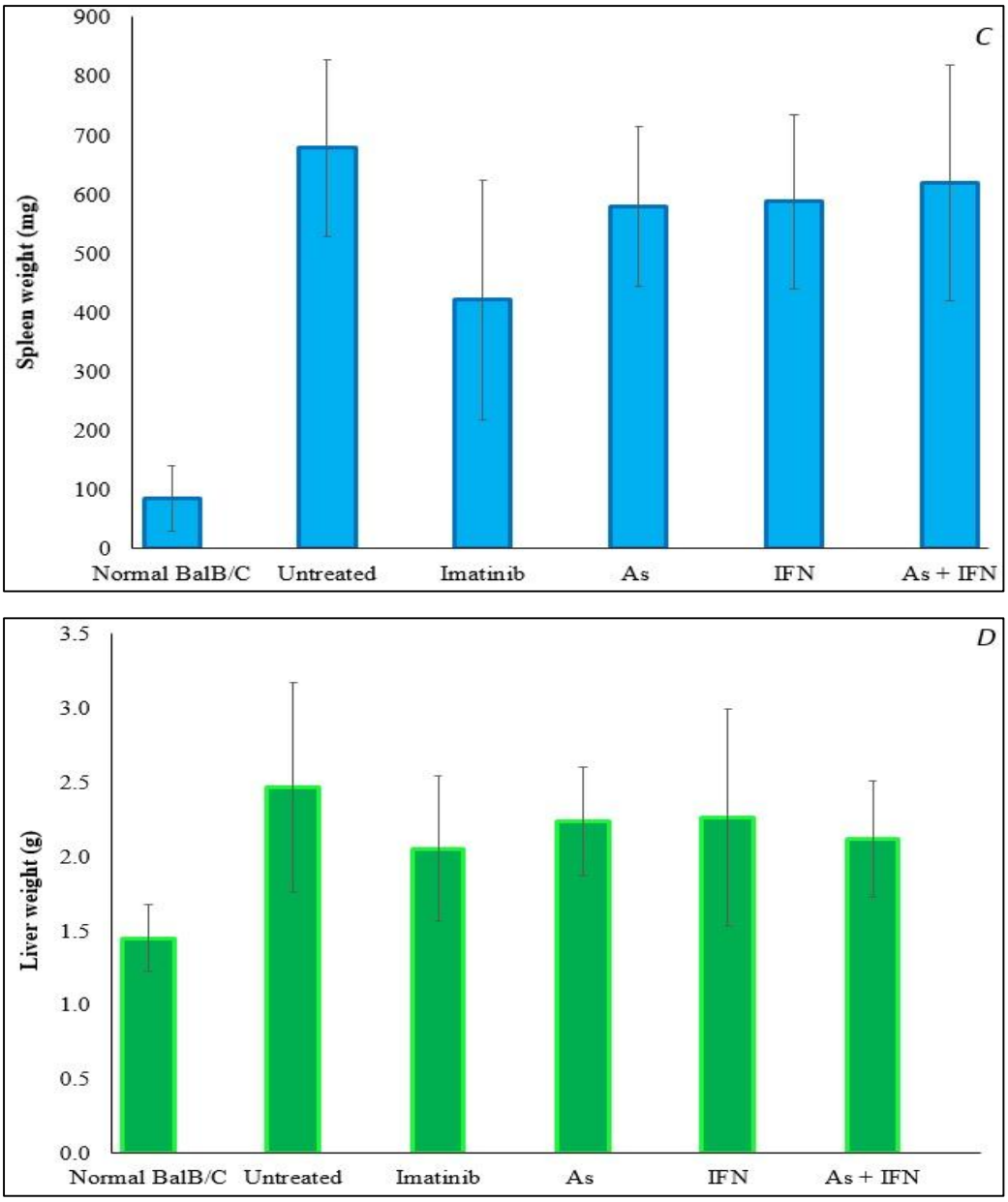


Figure 29 (*C* and *D*): Effect of ATO/IFNα treatment on spleen and liver weight.

Graph (A) represents the effect of 2-weeks treatment 5 days post transplantation on white blood cell count neutrophil count, and lymphocytes. Graph (B) represents the percentage of GFP positive cells in blood samples taken from primary mice. Graph (C) represents the weight of the spleen of primary recipients. Graph (D) represents the weight of the liver of primary recipients. Error bars shows standard deviations.

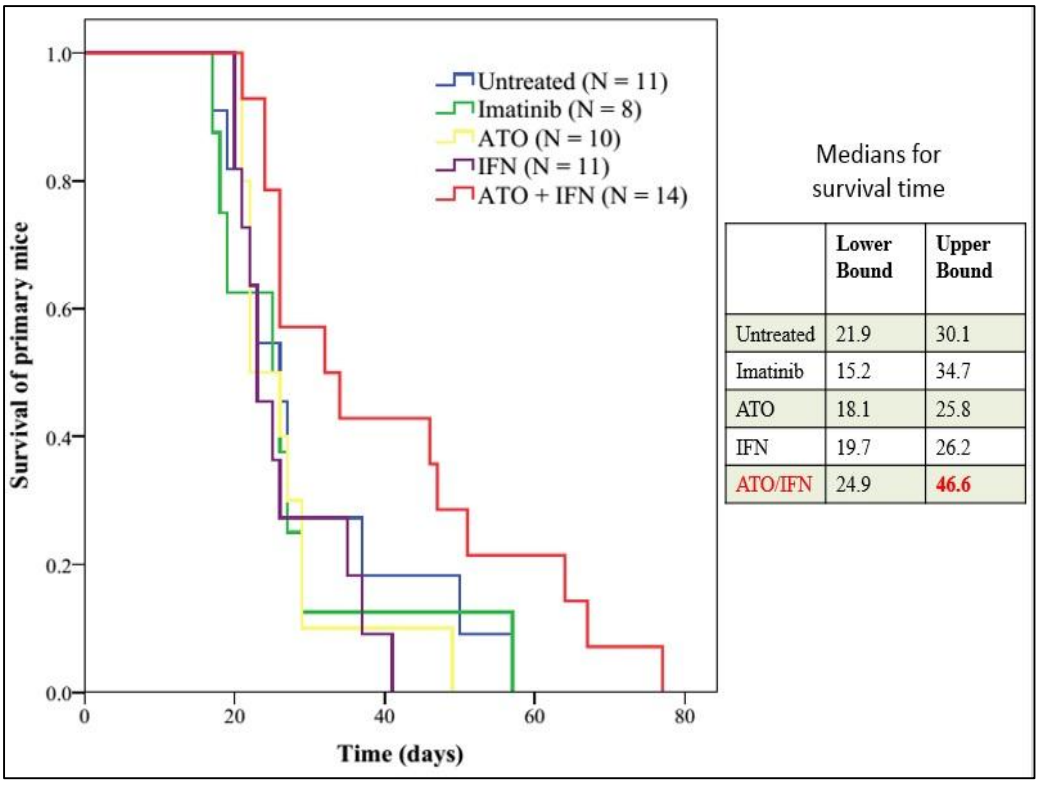


Figure 30: Kaplan–Meier plots demonstrating survival for T315I-CML primary mice.

Arsenic/IFNα treatment prolongs survival in T315I-CML mouse model. Kaplan–Meier plots demonstrating survival for T315I-CML primary mice.

Chapter IV

Discussion

The aim of this work is to investigate the effect of ATO/IFNα in imatinib-resistant and BCR-ABL T315I CML preclinical models.

Imatinib, the standard care of therapy for CML patients is not a curative drug since it cannot eradicate CML stem cells, which likely account for the relapse of CML patients following discontinuation of therapy [76, 77]. Moreover, although, imatinib is seen to induce long-term control of the disease in most CML patients, it is unable to be effective in imatinib-resistant patients including those suffering from a famous and most common tyrosine kinase domain mutation known as T315I mutation. CML patients harboring the T315I mutation, are resistant to all first and second generations TKI, and only sensitive to ponatinib, a third generation TKI [109]. However, ponatinib has some toxicity limitations and hence, ponatinib-treated patients should stay under strict observations at all times [109]. Moreover, there is recent evidence to demonstrate the emergence of ponatinib-resistant compound mutants, with more specifically, T315I inclusive compound mutants, which confer resistance to all TKI [109]. This stresses the need for the development of alternative and curative approaches that specifically eradicate T315I leukemic and CML stem cells [50].

We recently showed a potential curative role for ATO/IFNα combination in BCR-ABL wild type CML preclinical models [210]. In vitro studies demonstrated that ATO/IFNα treatment synergistically inhibited proliferation and triggered apoptosis in CML cell lines and reduced the clonogenic activity of primary CML cells derived from newly diagnosed CML patients, likely through inducing SUMO-dependent PML degradation [210]. Moreover, in vivo studies on wild-type CML murine model revealed survival prolongation in primary and secondary mice. Importantly, 1/3 of the secondary mice did not develop the disease post transplantation, and this reflects a specific effect of ATO/IFNα combination on the activity of leukemic stem cells [210]. Importantly, the apoptotic effects of ATO/IFNα on CML cells lines are unlikely to be mediated by the

decrease in BCR-ABL level or activity. For that, we were interested to investigate the effect of ATO/IFNα combination on imatinib-resistant CML cells and the BCR-ABLT315I CML mouse model.

Interestingly, the concentrations that we used are pharmacological concentrations of ATO and IFNα and both drugs are already used clinically. Specifically in ATL, ATO/IFNα combination has a greater clinical efficacy with acceptable toxicity [50].

In this study, our vitro data on imatinib-resistant CML cell lines, Ar230-R and K562-R cells, showed a clear growth inhibition in both cell lines treated with ATO/IFNα combination using achievable pharmacological concentrations of both drugs (ATO at 1 µM or 2 µM and IFNα at 100 IU/ml or 1000 IU/ml). In addition, cell cycle analysis demonstrated the induction of S+G2 M cell cycle arrest in both cell lines treated with the combination. Moreover, ATO/IFNα treatment induced apoptosis in imatinib-resistant CML cell lines that was accompanied by loss of mitochondrial membrane potential. This is in accordance with previous studies that have shown the implication of mitochondria in arsenic-induced apoptosis in several models [215].

Remarkably, our in vivo data showed that ATO/IFNα resulted in an increase in the survival of primary BCR-ABL T315I leukemic mice. The effect on the survival can be more significant by increasing the sample size and prolonging the treatment period to 3 weeks, since better results were achieved after 3 weeks ATO/IFNα treatment in wild type CML mouse model compared to 2 weeks of treatment [210]. On the other hand and as expected, imatinib had no effect on the survival of the mice. Surprisingly, imatinib-treated mice showed a decrease in the CBC count. This probably indicates that imatinib might have BCR-ABL independent effect in BCR-ABL T315I cells [84].

Notably, the effect of ATO/IFNα treatment, in vivo, on BCR-ABL T315I mice, seems to be more effective than on imatinib-resistant CML cell lines. It is important to note that the mouse model is exclusively dependent on T315I mutation, however the mechanisms of resistance in imatinib-resistant CML cell lines is not yet clear. This might probably include BCR-ABL or MDR overexpression, activation of several kinases, and the involvement of heat shock proteins [84, 216].

The mechanism of action of ATO/IFNα is not yet clear. ATO has been widely used in the management of patients with hematological malignancies such as APL [217, 218] and ATL [219]. Interestingly, ATO and/or IFNα were previously shown to affect leukemia initiating cells activity in APL and ATL [163, 212]. In those conditions, these drugs altered posttranslational modifications (sumoylation and/or ubiquitination) and accordingly affected the stability of oncogenic drivers: arsenic degrades PML-RARA, while IFNα/arsenic combination degrades the viral oncoprotein Tax [163, 220, 221]. Arsenic was also shown to down regulate PML that forces CML leukemic initiating cells to enter the cell cycle making them more sensitive to chemotherapy [222]. Recently, arsenic was shown to inhibit Hedgehog pathway in several cancers [223]. Interestingly, Hedgehog activation occurs in CML and increases with CML progression [224]. Our preliminary data showed that ATO/IFNα inhibited the transcriptional activity of Gli 2, a downstream protein of this stem cell renewal pathway.

On the other hand, IFNα has long been used as a therapy for cancer, particularly for CML, owing to its growth inhibitory and immune modulatory activities. IFNα induces hematologic and cytogenetic remissions and interestingly, improved outcome was reported with pegylated interferon and imatinib raising the possibility that IFNα might sensitize CML stem cells to imatinib [225]. Indeed, IFNα was shown to induce the exhaustion of normal hematopoietic stem cells [205]. Interestingly, it was recently reported that IFNα monotherapy induced a sustained deep molecular remission in a CML patient harboring the T315I mutation [226].

Since all these data proposed the specific effect of ATO and/or IFNα on HSC and LSC activity, we are currently carrying on serial transplantation assay to confirm whether in the BCR-ABL T315I CML mouse model, ATO/IFNα is specifically targeting leukemia initiating cells activity as it did in the BCR-ABL wild type CML mouse model [210].

In our study we had some limitations including the fact that BCR-ABL T315I CML mouse model is a new and not well-characterized model. For that, we need to include more mice in the future experiments to characterize this mouse model and the effect of our treatment on this mouse model. Moreover, imatinib-resistant CML cell lines used in these experiments are not T315I CML cell lines. Importantly, till now there

is no clear explanation about their mode of resistance. This is important since we found out that, sometimes, these cells are likely to lose their resistance in long-term culture. In addition, we need to do statistical analysis on all our results.

In conclusion, our data showed a preclinical efficacy of ATO/IFNα in preclinical models of imatinib-resistant preclinical models. Our promising data encourages further studies to elucidate the mechanism of action of ATO/IFNα. Moreover, if the preclinical efficacy is confirmed in additional experiments, this will hold a promise of this combination to be translated to the clinic.

Perscpectives

We are currently investigating the mechanism of action of ATO/IFNα combination in more details, including its effect on BCR-ABL and its substrates in the imatinib resistant CML mouse model and cell lines. We are also studying the effect of the ATO/IFNα combination on the hedgehog pathway. And we are performing the serial transplantation assay to confirm the effect of ATO/IFNα on leukemic stem cell activity in BCR-ABL T315I CML mouse model.

In the future, we aim also to investigate the effect of ATO/IFNα 3 week treatment. Moreover, our perspective is also to investigate the effect of sequential treatment of ATO/IFNα with Ponatinib on BCR-ABL T315I CML mouse model.

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