ORIGINAL ARTICLE
CELLULAR AND MOLECULAR RESPONSES OF SAUDI CHRONIC MYELOID LEUKAEMIA PATIENTS TO IMATINIB (STI-571): TEN YEAR EXPERIENCE


College of Applied Medical Sciences, Salman bin Abdulaziz University, Al-Kharj, *Internal Medicine Department, College of Medicine, University of Dammam, **Pharmacy Department, MoH, Riyadh, ***Biochemistry Department, KAMC, Riyadh, †Military Hospital, Riyadh, ‡Medical College, Northern Borders University, Arar, Saudi Arabia

Background: The cyto-genetic hallmark of chronic myeloid leukaemia (CML), the Philadelphia chromosome (Ph), is the first consistent chromosomal abnormality that has been associated to a certain cancer type. In CML, Philadelphia chromosome is present leading to resistance to cell death and rapid proliferation. The aim of this study is to evaluate the different responses, toxicity and survival of Saudi CML patients to imatinib mesylate. Methods: All newly diagnosed CML patients who were treated with imatinib were included in this study. We investigated haematological, and molecular and cyto-genetic responses by CBC, FISH and RT-PCR respectively. Cell proliferation and apoptosis were assayed using AUC and TUNEL respectively. Results: Of the 12 cases, 9 (75%) were males and 3 (25%) were female. Four (33%) of the cases were diagnosed incidentally and 8 cases (67%) presented mainly with fatigue (75%), fever (58%), and splenomegaly (83%). Signs of bleeding and rashes were rare at presentation. The majority of patients had low risk (8.67%), and 33% had intermediate risk; but none of them had high risk CML. At the last follow up, 11 (92%) were in remissions. One patient (8%) was in remission after 3 years, 4 (33%) were in remission after 6 years, one was in remission after 7 years and 5 (42%) were in remission after 10 years. Only one patient had incomplete major molecular response (MMR) to imatinib after 12 years. The majority of the patients (10, 83%) were in MMR after 6 years and 42% of them were in MMR after 10 years of therapy. Adverse effects of imatinib were not reported by the patients. Imatinib treatment resulted in the reduction of proliferation and induction of apoptosis of CML CFU-GM cells. Conclusion: Imatinib mesylate is capable of treating Philadelphia chromosome-Positive CP-CML without any adverse effects.

Keywords: Imatinib, Philadelphia, CML, responses

INTRODUCTION
The cyto-genetic hallmark of chronic myeloid leukaemia (CML), the Philadelphia chromosome (Ph), is the first consistent chromosomal abnormality that has been associated to a certain cancer type. The Ph chromosome was first reported by Peter Nowell and David Hungerford in the University of Pennsylvania in Philadelphia, United States after studying peripheral blood samples of two CML patients.¹ CML arises as a consequence of a rare mutational event resulting in a reciprocal translocation between the long arms of chromosomes 9 and 22. Approximately 90-95% of CML patients are Ph chromosome positive in cytogenetic analysis of the bone marrow samples.²,³

CML is an acquired clonal haemopoietic stem cell disease, with a chronic phase (CP), an accelerated phase, and a blast crisis phase (BC).⁴ Understanding the key cellular and molecular mechanisms involved in the BC phase of CML represents a vital goal in patient management because, once BC has been reached, treatment becomes extremely difficult, and prognosis poor.

Anti-apoptotic defects,⁵,⁶ high levels of proliferation,⁷⁸,¹⁰ insensitivity to negative regulators¹¹,¹² and defects in the adhesion mechanisms¹³–¹⁵ are considered to result from expression of the BCR-ABL fusion protein.¹⁶–¹⁷ These defects are therefore likely to be responsible for CML myeloid expansion.

Imatinib, (formerly STI-571, also called imatinib mesylate) is a rationally designed TKI which gained a UK license in 2001. It is a tyrosine kinase inhibitor that preferentially blocks the activity of ABL tyrosine kinases, in particular p210BCR-ABL¹⁸ and is the first targeted therapy for patients with CML and other tumours.¹⁸–²¹ It may lead to the inhibition of the signalling cascades downstream of BCR-ABL and inhibition of cell proliferation.²²–²³

Clinical experience has taught us the responses of CML patients may be broadly categorised into three groups according to whether they achieve a complete cytogenetic response (no BCR-ABL transcripts detectable by RT-PCR) or a haematological response (reduction in the white cell count):
1. Complete cyto-genetic responders. In these patients the Ph+ clone will be suppressed and haemopoietic cells in the blood and bone marrow will be Ph- and normal. This result suggests that CML clone was sensitive to imatinib and that there was sufficient
normal haemopoiesis to sustain haemopoietic activity.  
2. Haematological responders. In these patients the white blood cell count will fall in response to imatinib, but no cytogenetic improvement will appear. This implies that the CML clone was sensitive but that there was insufficient normal haemopoiesis to sustain cytogenetic response.  
3. Non responders. In these patients the white cell count does not respond fully to imatinib therapy, indicating that the CML clone is insensitive to this drug.  

This study focuses on the clinical presentation, haematological response, molecular response, survival and adverse effects of imatinib mesylate at the last visit of CML patients at our institution.

**MATERIAL AND METHODS**

Twelve patients, nine males and three females in the age group of 17–65 years who were diagnosed as chronic phase of chronic myeloid leukaemia (CP-CML) at our hospital since 2001 who were treated with imatinib mesylate. All patients were diagnosed according to clinical symptoms and signs, peripheral blood smear findings, bone marrow aspiration and biopsy findings and reverse transcriptase quantitative polymerase chain reaction (RT-PCR). The dose of imatinib administered was 400 mg/day orally. They were followed closely at the oncology clinic during the period of 2002–2012. During this period, clinical examination, complete blood count (CBC) and peripheral smears were done every 3 months. The RT-PCR was done at the last visit. The patients were considered as having clinical response if they were free of symptoms and signs of CML. They were considered as having responded at the haematological level if they achieved normal haemoglobin, leucocyte, and platelet indices at the last visit as shown in Table-1 and 2.

Peripheral blood and bone marrow samples obtained from patients in CP-CML who were scheduled to receive imatinib therapy. Samples obtained before treatment and after treatment from those patients who become complete cytogenetic responders. Normal control materials obtained as bone marrow from individuals who are donating cells for allogeneic transplantation and as blood from normal volunteers. Written informed consent and Research Ethics Committee approval was obtained in all cases.

Monitoring of this disease quantitated using Real-Time PCR in the forthcoming protocol, using IgH and TCR genes as genomic targets. Reverse transcriptase standardised in our laboratory for the p190 and p210 fusions. This technique is used to monitor all transcriptase standardised in our laboratory for the p190 and TCR genes as genomic targets. Reverse transcriptase quantitative polymerase chain reaction (RT-PCR) was done at the last visit. The RT-PCR was done again in 10 ml MM and gently but thoroughly re-suspended in 1 ml of MM buffer.

Apoptosis, where appropriate detected using TUNEL and flow cytometry (Annexin-V labelling). Fluorescence in situ hybridisation (FISH) analysis of CD34+ cells and of colonies grown in vitro was done. This technique has been used to discriminate between Ph+ and Ph- cells at different stages of maturation and colonies grown in myeloid colony assay. CD34+ cells were separated using MiniMACS magnetic immuno-affinity columns as directed by the manufacturer (Miltenyi Biotec). Cells and colonies will be transferred into multicompartimentalised slides and air dried by evaporation. The cells will then be probed using a mixture of BCR sequences labelled with Spectrum Green and ABL sequences labelled with Spectrum Orange (Vysis) as directed by the manufacturer. Ph+ cells will be identified by the coincidence of green and orange signals. FISH combined with immunophenotyping will be used to determine the relative frequencies of Ph+ and Ph- cells in CD34+ cell subcompartments (CD33±, CD38±, HLA-DR±). Finally CD34+ cells will be stained for proliferating cell nuclear antigen (PCNA) and probed by FISH to measure the frequencies of quiescent versus proliferative Ph+ and Ph- cells.

**Preparation of Human cells to cell culture:**

Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard). After washing with HBSS (GibcoBRL), cells were resuspended in HBSS. To calculate the concentration of nucleated cells, the sample was diluted with 3% acetic acid to lyse red blood cells and an aliquot placed in a haemocytometer counting chamber. The cell concentration was adjusted to 10×10⁶/ml with 15% FCS in minimal essential medium (MEM) (GibcoBRL). The cell suspension was placed in a 25 Cm² plastic flask for 2 hours incubation at 37 °C in humidified 5% CO₂ in air to remove adherent monocytes and macrophages. Non adherent MNCs were harvested for culture.

Purification of human CD34+ cells CD34+ cells were separated using MiniMACS magnetic immuno-affinity columns as directed by the manufacturer (Miltenyi Biotec). Cells from pooled day 7 CFU-GM were incubated for 15 min at 4 °C with 100 µl reagent A1 (blocking FcR), 100 µl of reagent A2 (hapten-conjugated anti CD34+ Mab) and 300 µl of MiniMac buffer (MM) containing calcium and magnesium-free PBS (GibcoBRL). 0.5% human serum albumin (Immuno AG) and 5 mM EDTA (Gibco BRL). Following a single wash in 10 ml MM, cells were re-suspended with 100 µl reagent B (anti-hapten Mab conjugated to beads) and 400 µl of MM buffer and incubated for 15 min at 4°C. Cells then were washed again in 10 ml MM and gently but thoroughly re-suspended in 1 ml of MM buffer. The labelled cells
were loaded onto the affinity column held in a magnet and CD34\(^+\) cells washed through with 4×0.5 ml of MM buffer. After removal from the magnet, the CD34\(^+\) cells were vigorously expelled from the column with 1 ml MM buffer. The purity is about 85–98%, confirmed by flow cytometry.

All recombinant cytokines were obtained from First Link. The concentration of each cytokine used was optimized in the laboratory.

Non-adherent MNCs were mixed with 3 ml of methylcellulose (MC) (1×10\(^5\) cells/ml) (Stem Cell Technologies) containing 10% FCS and supplemented with recombinant human cytokines {IL-3 (5 ng/ml), G-CSF (100 ng/ml), GM-CSF (1 ng/ml) and SCF (20 ng/ml)}. After being mixed well, 1 ml aliquots were plated out into three 35 cm\(^2\) diameter petri-dishes. The dishes were then incubated at 37 °C in humidified 5% CO\(_2\) in air for 7 days. Colonies consisting of 50 cells or more were scored under an inverted microscope.

After 7 days of incubation, 120 colonies consisting of 50 cells or more were plucked individually from MC using a sterile eppendorf pipette and thoroughly dispersed in separate wells of a 96 well plate, each containing 100 ml of MC plus FCS and supplemented with the four recombinant human cytokines. The surrounding wells were filled with sterile water to prevent evaporation during incubation. Plates were incubated for a further 7 days at 37°C in humidified 5% CO\(_2\) in air. Then each well was scored for secondary colonies of more than 50 cells. The results were analysed by using Microsoft Excel version 5.0. They were plotted as the cumulative distribution of the proportion of primary CFU-GM producing more than n secondary colonies. Both axes are expressed on a logarithmic scale. This step allows good data fit, so that the Area-Under-the Curve (AUC) can be measured using the Trapezium Rule. This is done because the distribution of secondary colonies is highly skewed rather than being normally distributed. Therefore the median and not the mean is the correct value to use. However, for cases where more than 50% of the replated colonies do not yield secondary colonies, the median would be zero and uninformative. The AUC is calculated to provide an overall measure of progenitor cell self-renewal capacity. The AUC is calculated to provide an overall measure of progenitor cell self-renewal capacity. The sensitivity to Imatinib is given by the ratio between the AUC of the control culture and the AUC of the culture incubated in the presence of Imatinib. Data from the Ph- colonies will be compared with data from normal blood and bone marrow controls. In the case of complete cytogenetic responders we will study samples obtained by mobilisation, in addition to blood and bone marrow, and compare results with samples from normal stem cell donors who have been subjected to the same mobilisation regimen.

Cells were washed twice with phosphate-buffered saline (PBS) buffer and lysed at 4×10\(^5\) cells/ml in cell lysis buffer (20 mM HEPES, pH 7.4, 0.25% NP-40 containing protease inhibitor cocktail; Boehringer Mannheim, Indianapolis, IN) for 10 minutes on ice. Equal amounts of lysate (equivalent to 5X10\(^3\) cells) were subjected to SDS-PAGE to 12% polyacrylamide gels. Proteins were transferred to Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) membranes and reacted with first antibody for 2 hours at room temperature. After they were washed, membranes were probed with a horseradish peroxidase-conjugated secondary antibody and reacted with ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Anti-β-actin blot was made in parallel as a loading control. Signals were detected by a Phosphor Imager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA) and quantified by Scion Image software (Scion, Frederick, MD). Results were expressed as 1st Ab/β-actin ratios.

Statistical analysis was carried out using Microsoft Excel spreadsheet and the Stat View SE+ graphics software. The probability of a significant difference between groups was determined by Mann-Whitney U test and Wilcoxon Signed rank test. Graphs were plotted using Cricket graph graphics package. Correlation was calculated using Spearman Rank test. The AUC was calculated using a Microsoft Excel spreadsheet. All software was run on a Macintosh computer.

RESULTS

Nine (75%) out of twelve patients with Philadelphia-chromosome positive chronic phase CML were males and 25% were females (Table-1). The most common symptoms and clinical signs with which the patients presented were fatigue 9 (75%), fever 7 (58%), rash 3 (25%), bleeding 2 (17%) and splenomegaly 10 (83%). Four (33%) of patients were diagnosed incidentally. Eight (67%) of the patients had low risk disease, 4 (33%) of them had intermediate risk and none had high risk (WHO scoring system). Five (42%) of patients were in clinical, complete haematological and major molecular responses after 10 years, 1 (8%) after 7 years, 4 (33%) after 6 years and 1 (8%) after 3 years of therapy. One (8%) patient had clinical and complete haematological remission by 10 years of treatment but failed to have MMR. Imatinib mesylate was safe in our patients and no side effects were reported (Table-2).

We performed dose-response curve for the imatinib to determine the highest concentrations of imatinib that did not have suppressive effect on the colony formation (data not shown). Then, we used these obtained concentrations to study its effects on the proliferation of primary colonies. There is a significant reduction in the AUC of those treated with Imatinib
compared to untreated controls ($p=0.05, n=10$) (Figure-1 and 2). Interestingly, imatinib responses have no effect on NBM CFU-GM as it did for CML CFU-GM.

Use of in vitro assay systems, demonstrated clear differences between normal and CML cell proliferation, adhesion and responses to imatinib and IFN-α. The AUC in CML is significantly higher than normal, and is significantly reduced by imatinib or IFN-α compared to normal, but there are wide variations in both measurements. The tyrosine kinase inhibitor ‘Imatinib, STI571’ selectively inhibits the tyrosine activity of ABL and BCR-ABL proteins and the formation of CFU-GM colonies from CML patients. We found also that imatinib has a greater and more selective effect on the replating ability of CFU-GM from CML patients, than it has on primary colony numbers. In contrast to the suppression seen in CML CFU-GM, the replating ability of normal CFU-GM was significantly enhanced at low concentration of the compound (0.1 μM) when the differential effect was greater (Figure-3).

In some patients, increased amounts of lymphocytes were observed in follow-up samples during imatinib therapy. The morphology of lymphocytes varied in size and shape and also included immature forms. At the time of diagnosis none of the patients had increased amounts of lymphocytes in the bone marrow as the mean percentage of lymphocytes was 3%. After 9 months of imatinib therapy, the bone marrow cellularity had decreased and was normal in 9/12 patients. The median percentage of lymphocytes was 7%, but there was a marked variability between different patients. At the time of diagnosis, the CML patients had decreased number of B cells in the bone marrow compared to normal control values (10% of lymphocytes vs 29%), and no immature or maturating forms were detected. Patients with suboptimal response to imatinib also had decreased number of B cells in the bone marrow, whereas patients having optimal response and bone marrow lymphocytosis had normal or increased amounts of B cells. In T cells the CD4/CD8 ratio was normal and the proportion of regulatory T cells (Tregs) in bone marrow was also comparable in different groups. The numbers of DC were comparable to normal levels in patients with optimal response to imatinib treatment.

Baf3 cells were exposed to imatinib (20 nM) for 24 hours and then lysates prepared for Western blot. Following SDS-PAGE electrophoresis, the samples were probed with anti-pCrkl and anti-actin. Crkl is an adaptor protein which plays an important role in BCR ABL mediated signal transduction. By this technique we demonstrated that in Baf3 cells imatinib exposure appeared to be associated with a decrease in BCR-ABL activity. BCR-ABL activity was effectively reduced with exposure to 150 nM imatinib for 24 hour for comparison.

Patient samples CML CD34+ cells were cultured in GM mix in the presence and absence of imatinib. At each time point the viable cell count and percentage of apoptotic cells were calculated. With increasing concentration of imatinib there was an increase in the percentage of apoptotic cells staining positive for annexin-V alone (early apoptosis) and both annexin-V and 7-AAD (late apoptosis) (Figure-4).

**Table-1: Demographic characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
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<td>Total numbers of patients</td>
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<td>Male/female</td>
<td>9/3</td>
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<tr>
<td>Age: range</td>
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<td>Fever</td>
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</tr>
<tr>
<td>Fatigue</td>
<td>9</td>
</tr>
<tr>
<td>Bleedings</td>
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<td>Rash</td>
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<td>Splenomegaly</td>
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<tr>
<td>WHO score</td>
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<tr>
<td>Low risk</td>
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</tr>
<tr>
<td>Intermediate risk</td>
<td>4</td>
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<td>High risk</td>
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**Table-2: Response to imatinib mesylate therapy**

<table>
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<tr>
<th>Patients</th>
<th>Clinical response</th>
<th>CHR</th>
<th>MMR</th>
<th>CMR</th>
<th>Duration of treatment</th>
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</table>

CHR=complete haematological response, CMR=complete molecular response, MMR=minor molecular response

**Figure-1: Self-renewal capacity (AUC) of CFU-GM from CML patients treated with STI-571**

The results are compared to untreated cells. There is a significant reduction in the AUC of those treated with STI-571 compared to untreated controls (Wilcoxon Signed-rank test).

**Figure-2: Self-renewal capacity (AUC) of CFU-GM from NBM controls treated with STI-571**

The results are compared to untreated cells. There is no significant difference in the AUC of STI-571 compared to untreated controls (Wilcoxon Signed-rank test).
Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the pluripotent stem cells with an incidence of 1 per 100,000 in the west 24,25 whereas, in Saudi Arabia the true incidence is not known. All leukaemia in general account for 6.2% of all newly diagnosed cancers in year 2004, and only 4.6% and 6.7% were due to CML in male and female respectively. 26 The median age of patients suffering of CML is 67 years in the west with slight male predominance 27, however, in our study the median age was 39 years with male predominance.

The old conventional chemotherapy of CML has been either Busulphan or hydroxuryrea with many adverse effects, median survival of less than 3 years, haematological remission of less than 80% and no cytogenetic remission. 24,28 Allergic stem cell transplantation is the only curative treatment for CML but not always feasible or available with high mortality rate and adverse effects. Interferon Alfa was the treatment of choice before tyrosine kinase inhibitors (TKI).

Nowadays, the standard frontline therapy of CML patients is imatinib 400 mg daily. 29 The inhibition of BCR-ABL tyrosine kinase is predictable, selective and effective therapy for Philadelphia chromosome positive CML. The adverse reactions are less and manageable compared to the old options of therapy.

In the present study, we investigated whether imatinib treatment behave in the similar manner as other populations. Twelve patients were followed up for 10 years. The results demonstrated that none of our patients complained of any adverse effect even though the duration of therapy was long, ranging from 3–10 years and 90% had a duration more than 6 years. These results are on line with many local reports from Saudi Arabia. 30–32

Studies have shown that after 6 years of follow-up, 86–88% of patients with Ph+ CML who were treated with imatinib mesylate remained in MMR, which was defined as a 3-log reduction in the quantity of the Bcr-Abl transcript and has emerged as a new target response to achieve and is gaining acceptance as a surrogate for long-term benefit in clinical studies. 33,34

In our study, even though small one, 83% of our patients are in MMR after 6 years and 42% are in MMR by 10 years of treatment and none of them has complained of side effects or has discontinued imatinib mesylate therapy. Our study is on agreement with several studies which showed that an overall survival rate of 95.2% after 8 years. 35,36

To investigate whether the self-replication of CML CFU-GM could be reduced to the levels of NBM CFU-GMs, STI571 was incubated at various concentrations. There was a decrease in the CML CFU-GMs replication to the levels seen in NBM, But did not affect the NBM replication. These results are consistent with Gordon’s group. 37–39

These findings showing that imatinib suppresses progenitor cell proliferation, as reflected by the AUC obtained in the colony replating assay, by CML but not by normal CFU-GMs replication to the levels seen in NBM. The idea that different downstream signalling pathway may be activated to different extents and committedly inactivated by treatment with imatinib is supported by our preliminary findings on the effects of pathway inhibitors in individual patients. Our data have implicated the PI3-kinase pathway and suggest that downstream target (e.g., ERK and p70) are differently affected in different patients. These initial results support imatinib as a less toxic and orally administrated alternative to IFN-α therapy of CP-CML. Two reports showed that STI571 induced apoptosis in CML cells30,41 as our results revealed.

It could be inferred that the presence of fully functional genes regulating both cell cycle and apoptosis, will maintain the balance between the rate of cell division and apoptosis of any population in vivo. Therefore, any malfunction or loss of any one of the above mentioned genes, may lead to an increase in their self-replication.42,43

CONCLUSION

Imatinib mesylate is effective and safe even after 10 years of treatment and follow-up of patients suffering of Ph+ CML.
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Address for Correspondence:
Dr. Faris Q. Alenzi, Professor of Immunology, Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Salman bin Abdulaziz University, PO Box 422 Al-Kharj 11942, Saudi Arabia.
Email: fqalenzi@ksu.edu.sa