

CO-RELATION OF P-GLYCOPROTEIN AND PLASMA IMATINIB IN IMATINIB RESPONDERS AND NON-RESPONDERS PATIENTS WITH CHRONIC MYELOID LEUKAEMIA

LOKESH BAGARI NAGAPPA¹, ASHWATHY VARADARAJAN THUNDAKATTIL^{2*}

¹Department of Pharmacology, All India Institute of Medical Sciences, New Delhi-110608, India. ²Faculty of Medicine, Manipal University College Malaysia-75150 Melaka, Malaysia

*Corresponding author: Ashwathy Varadarajan Thundakattil; *Email: ashwathy.vt@gmail.com

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ABSTRACT

Objective: To measure and compare P-glycoprotein (P-gp) expressions in imatinib responders and non-responders with chronic myeloid leukemia-chronic phase (CML-CP) and correlate with plasma imatinib levels.

Methods: Patients were classified into two groups based on their haematological and cytogenetic responses to imatinib: responders and non-responders. Liquid chromatography-mass spectrometry was used to measure plasma imatinib levels, while flow cytometry was used to evaluate leucocyte P-gp expression.

Results: The median plasma imatinib trough levels in non-responders were 496 (217-3150) ng/ml compared to 2245 (454-4270) ng/ml in the responders, which was statistically significant ($p=0.0003$). The proportion of patients expressing P-gp in granulocytes was higher in the non-responder group than in the responder group (75% vs. 62.5%). The ratio of mean fluorescence intensity (RFI) revealed that non-responders had higher median P-gp expression than did respondents MFI (1.16(1.06-1.50) and 1.12(1.01-1.38), respectively; $p = 0.2307$). In both groups, there was a negative correlation between P-gp expression and plasma imatinib trough levels (-0.4384 vs.-0.2848).

Conclusion: Imatinib median plasma trough levels in non-responders were considerably lower. This was highly supported by P-gp expression in granulocytes, which is inversely related to imatinib plasma trough levels; however, the difference was not statistically significant, which could be attributed to the small number of patients. This could be the cause of imatinib resistance in non-responder CML-CP patients, and P-gp levels should be evaluated to optimize treatment in patients who do not achieve hematologic or cytogenetic response.

Keywords: Imatinib resistance, CML, Plasma trough levels, P-glycoprotein

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INTRODUCTION

Pluripotent hematopoietic stem cells' myeloproliferation causes chronic myeloid leukaemia (CML) [1]. The Philadelphia (Ph) chromosome mutation (9; 22) (q34; q11), which causes the production and translation of the BCR-ABL protooncogene with tyrosine kinase activity, is the most common feature of CML [1-3]. The initial line of treatment for CML is imatinib, a tyrosine kinase inhibitor (TKI) [4]. By attaching to the ATP-binding pocket of the BCR-ABL protein, it prevents the signalling cascade required for the conversion of wild hematopoietic cells into CML cells [5]. However, in recent years, the main factor contributing to the failure of CML therapy has been imatinib resistance. Numerous imatinib resistance mechanisms, including gene mutations in the BCR-ABL kinase domain and overexpression of SRC kinase, have been described previously [5-9]. Studies have suggested that low plasma imatinib levels may be caused by the efflux of imatinib from leukemic cells through P-glycoprotein (P-gp), which is encoded by the transporter gene MDR1 and leads to imatinib failure [7, 8]. Some investigations, however, have shown that P-gp overexpression is unlikely to be a major driver of imatinib resistance [11, 12]. Therefore, there is a need for further exploration of the correlation between P-gp and imatinib resistance. We conducted this investigation to determine the involvement of leucocyte P-gp in imatinib therapy failure in CML patients.

MATERIALS AND METHODS

Study population

Participants in the research were people with CML-CP who visited the CML clinic in the medical oncology department at AIIMS, New Delhi, between February 2012 and December 2013. Adult patients diagnosed with CML according to NCCN standards and receiving imatinib medication therapy for the condition were included in the study [4]. Patients who met the following criteria were excluded: (i)

those with hepatic dysfunction, defined as two times the upper limit of normal for serum bilirubin, alanine transaminase, and aspartate transaminase; (ii) those with two times the upper limit of normal for serum creatinine; and (iii) patients who were pregnant women. Written informed consent was taken from all participants before their enrolment in the study.

Ethical statement

The study protocol was reviewed and approved by the institutional Ethics Committee, AIIMS, New Delhi committee (IESC/T-97/2.3.12). The study was carried out by the Declaration of Helsinki's rules and supported recommended standards of good clinical practice.

Study design

This was pilot research using a cross-sectional design. The sample size was calculated, and 40 patients were included in the pilot trial to determine the association between P-gp and imatinib resistance. Patients were split into two groups based on how well they responded to imatinib therapy: imatinib responders and imatinib non-responders. Patients who experienced a full hematopoietic response within three months of beginning imatinib therapy or a substantial cytogenetic response within six months of initiating the medication were considered imatinib responders. The lack of Philadelphia chromosome-positive cells in bone marrow cytogenetic research that used Giemsa banding analysis to analyse at least 20 metaphases was referred to as a complete cytogenetic response (CCyR). Patients who did not achieve the cytogenetic or haematological response stated above in the responder group were classified as imatinib non-responders [13].

Blood sample collection and plasma separation

Three ml of blood was collected from the antecubital vein of patients under aseptic conditions into an EDTA vial 24 h after the previous dosage of imatinib and before taking the subsequent dose of the

medication to estimate imatinib plasma trough levels. One millilitre of this blood sample was utilized to analyse leucocyte P-gp expression using flow cytometry, and the remaining two millilitres were centrifuged at 3000 rpm for ten minutes within an hour to obtain plasma. Within a month of storage at -80 °C, imatinib levels in the extracted plasma were calculated using liquid chromatography-mass spectrometry (LC-MS).

Extraction of imatinib from plasma by liquid chromatography-mass spectrometry

Before processing, all frozen plasma samples were allowed to thaw at room temperature. 50 µl of standards or samples were combined with 200 µl of extraction solvent (90% acetonitrile, 0.1% formic acid, and 250ng/ml homatropine as an internal standard). The mixture was vortexed for 1 minute before being centrifuged at 1000 rpm for 10 min. The resulting supernatant was subjected to analysis. The assay was carried out using a ZIC HILIC column (dimension of 50 mm X4.6 mm, Merck, Germany) with a flow rate of 0.5 ml/min and a photodiode array detector was used to estimate imatinib plasma trough levels.

Analysis of P-gp expression

A total of 5×10^5 leukemic cells were isolated from the peripheral smears of CML-CP patients and treated with Phosphate buffer solution (PBS)+1% Bovine serum albumin (BSA). The test sample was centrifuged for five minutes at a speed of 1500 rpm after incubation. After discarding the supernatant, cells were given two PBS washes over 15 min. Using 5 µl of Phycoerythrin (PE)-conjugated monoclonal antibody against P-gp from Becton Dickinson (USA), these leukemic cells were directly labeled. Following staining, cells were washed twice with PBS, fixed with 300 µl PBS+1% paraformaldehyde, and acquired using a flow cytometer. Based on forward and side scatter characteristics (FSC x SSC), leukemic cells were gated on granulocytes. The mean fluorescence intensity (MFI) of cells treated with anti-P-gp was divided by the MFI of cells not stimulated with monoclonal antibodies to produce the RFI [14]. The RFI limit for expression positivity (RFI= 1.1) was based on prior research utilizing the P-gp-negative CML K562 cell line and its vincristine-resistant variant, K562-Lucena.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, Chicago, USA) was used to analyze the data. Student t-tests were employed to assess the quantitative data of the imatinib responders and imatinib non-responder groups, while Mann Whitney was utilized to analyze

the sample that was not normally distributed. The Pearson correlation was performed to examine the association between P-gp and imatinib plasma trough levels. The information was presented as a median (95% CI). Statistical significance was defined as a p-value less than 0.05.

RESULTS

Baseline characteristics of the study population

The research included a total of 40 patients. In both groups, most patients (75% of responders and 68.9% of non-responders) were men. The age distribution of respondents and non-responders differed significantly (35 (22-50) years vs. 46 (26-67) years, $p=0.04$). Abdominal fullness was evident in 56.3% of responders and 62.5% of non-responders at the time of presentation, fever was present in 56.3% of responders and 50% of non-responders, and fatigue was present in 43.8% of responders and 56.3% of non-responders. Four individuals in the non-responder group had spleens that measured ≥ 20 cm. Hepatomegaly, on the other hand, is less noticeable than splenomegaly.

Assessment of haematological and cytogenetic parameters

The median haemoglobin level was 9.8 g/dl in the responder group and 10.55 g/dl in the non-responder group, respectively. The median platelet count was $220 \times 10^3/\text{mm}^3$ in the responder group and $378 \times 10^3/\text{mm}^3$ in the non-responder group, while the median total leucocyte count was $126 \times 10^3/\text{mm}^3$ ($12.2-309 \times 10^3/\text{mm}^3$) in the responder group and $140 \times 10^3/\text{mm}^3$ ($32.7-419 \times 10^3/\text{mm}^3$) in non-responder group. The median absolute neutrophil count among responders was $75.1 \times 10^3/\text{mm}^3$ ($9-147 \times 10^3/\text{mm}^3$) and $82.9 \times 10^3/\text{mm}^3$ ($24.1-167 \times 10^3/\text{mm}^3$), whereas the median basophil count was 2% among responders and 3% among non-responders.

The number of peripheral blasts among respondents was 2% compared to 0.5% among non-responders. The median myeloid to erythroid ratio in bone marrow was 8.5 (interquartile range: 1-33) and 12.19 (interquartile range: 1.2-25) in responders and non-responders, respectively. Myeloid linkage reveals two peaks, one for myelocytes and one for segmented neutrophils (fig. 1). Two-thirds of the patients exhibited fibrosis of the bone marrow. Before beginning the imatinib therapy, 12 patients in the responder group and 13 patients in the non-responder group were >90% Philadelphia chromosome-positive.

Detection of plasma trough level of imatinib

Individual plasma imatinib trough levels were quantified through liquid chromatography-mass spectrometry (fig. 2A-2D).

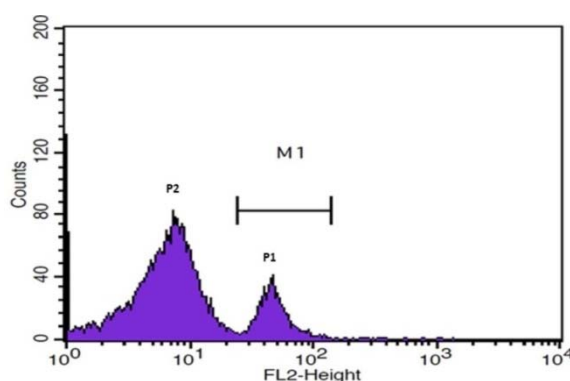
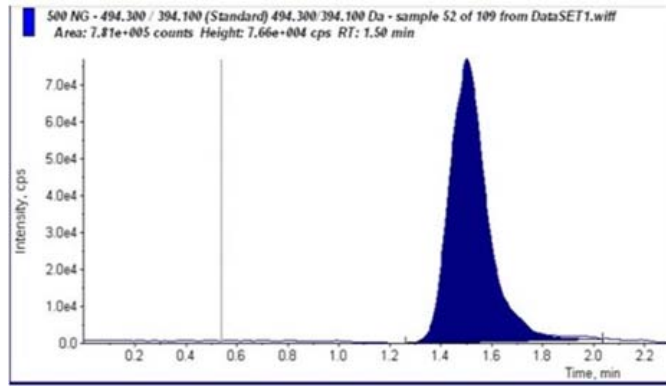


Fig. 1: Flow cytometric analysis of chronic myeloid leukaemia cells. Histogram plot shows different granulocytic cell populations from CML CP patients

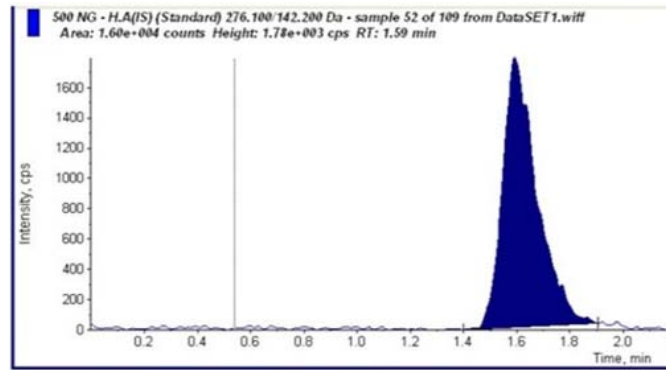
Table 1: Plasma trough level of imatinib in CML-CP patient's variable

	Imatinib responder	Imatinib non responder	p-value
Mean(ng/ml)	2174	819	0.0003
Median(ng/ml)	2245	496	
Range(ng/ml)	454-4270	217-3150	

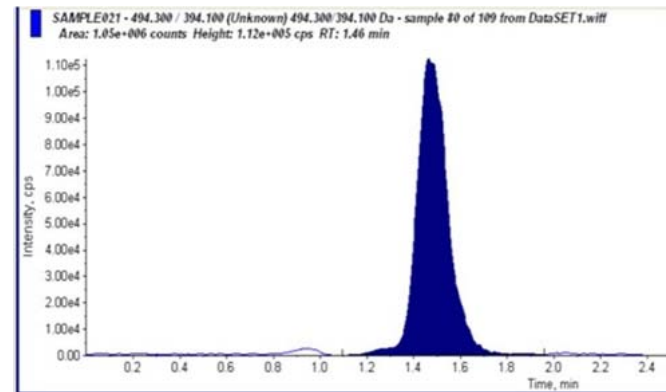
As indicated in table 1, the Median plasma imatinib trough levels in non-responder groups were significantly lower than those in the responder group (496 (217-3150) ng/ml vs.2245 (454-4270) ng/ml, $p=0.0003$).



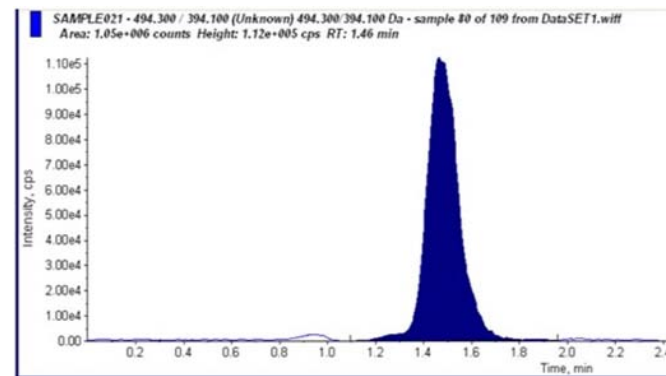
2A



2B



2C



2D

Fig. 2A: MRM chromatogram resulting from analyses of standard imatinib. 2B: MRM chromatogram resulting from analyses of internal standard. 2C: MRM chromatogram of human plasma spike with 500 ng/ml of imatinib. 2D: MRM chromatogram resulting from analyses of imatinib from patient plasma

Imatinib plasma trough levels were shown to vary amongst patients in both groups, with responder group variability being larger than non-responder group variability (fig. 3).

P-glycoprotein expressions in CML-CP patients

Flow cytometry was used to evaluate P-glycoprotein expression (fig. 4).

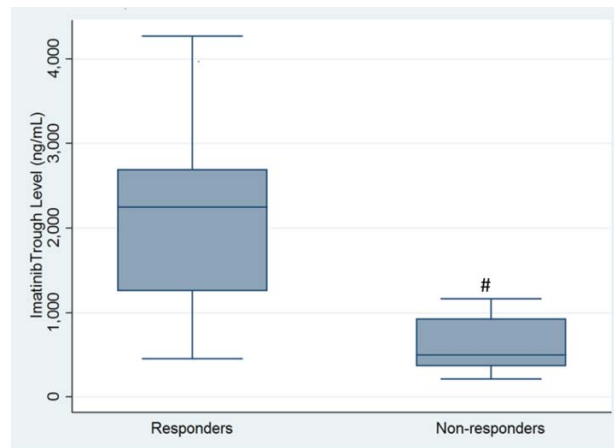


Fig. 3: Plasma trough levels of imatinib in responders and non-responders of CML-CP patients

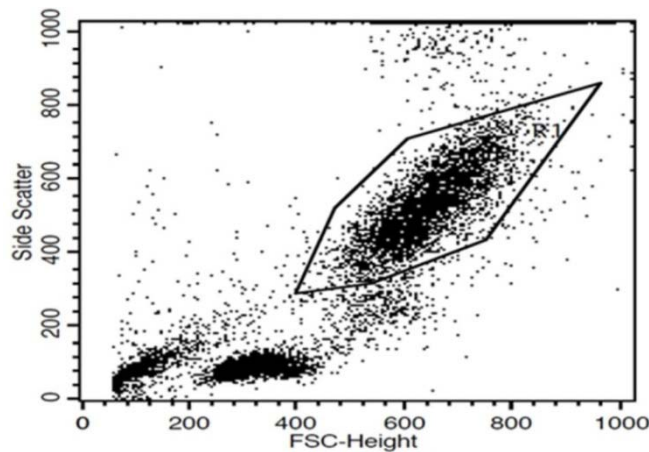


Fig. 4: Dot plot for analysis of P-gp expression in lymphocyte population of peripheral blood mononuclear cells from CML patients through flow cytometry

The proportion of patients who responded to imatinib was 60%, with a median fluorescence intensity P-gp expression of 1.12 (interquartile range: 1.01-1.38). The non-responders' mean P-gp expression was 1.16 (interquartile range: 1.06-1.50 MFI). P-gp expression was seen in 15 out of 20 (75%) patients who were imatinib nonresponders, as indicated in table 2. There was no statistically significant difference in the levels of P-gp expression between the two groups ($p=0.2307$).

Correlation between P-gp expression and plasma trough levels of imatinib mesylate

P-gp expression in leukemic cells and the plasma trough levels of imatinib in both groups were inversely correlated ($r=-0.2848$ in the responder group and $r=-0.4384$ in the non-responder group). However, neither group's statistical analysis of this association found it to be significant ($p=0.2851$ for the responder group and $p=0.0894$ for the non-responder group; table 3).

Table 2: P-glycoprotein expressions in granulocytes of patients with CML-CP

Variable	Responder	Non-responder	P value
Mean (MFI)	1.15	1.21	0.2307
Median (MFI)	1.12	1.16	
Range (MFI)	1.01-1.38	1.06-1.50	

Table 3: Correlation between P-gp expression and plasma trough levels of imatinib mesylate

Group	Observation	P-gp expression (MFI)	Trough levels of imatinib (ng/ml)	Spearman's rho p-value
Responder	Mean±SD	1.15±0.1368	2174.625±1086.205	$r=-0.2848$
	Median	1.12	2245	$p=0.2851$
	Interquartile range	1.01-1.38	454-4270	
Non responder	Mean±SD	1.21±0.1405	819±779.359	$r=-0.4384$
	Median	1.16	496	$p=0.0894$
	Interquartile range	1.06-1.50	217-3150	

DISCUSSION

Since imatinib was suggested by the NCCN guidelines as the first-line therapy for CML, the management of the disease has undergone a significant change [4]. The majority of circulating imatinib binds to plasma proteins, namely albumin and alpha acid glycoprotein (AGP), and the elimination half-life is around 18 h [15]. Imatinib is efficiently absorbed when taken orally. Tyrosine kinase inhibitors may have treated CML well, but imatinib resistance has prevented it from happening. At a dosage of 400 mg per day, we found that non-responders had substantially lower median plasma levels of imatinib than did responders (496 (217-3150) ng/ml vs. 2245 (454-4270) ng/ml, $p=0.0003$).

Some investigations have shown that imatinib resistance was depending on plasma drug concentration and was brought on by inadequate dosages, reduced drug influx via the hOCT1 transporter, or enhanced drug efflux brought on by greater P-gp expression [16]. In a trial by Larson *et al.*, patients experienced a full haematological response when plasma imatinib levels were >1000 ng/ml, and a poor haematological response was associated with low plasma levels of imatinib [15]. Previous reports also suggested that the patients with higher mean plasma imatinib levels of >1000 ng/ml experienced cytogenetic response [17]. Further studies discovered that patients with an imatinib plasma trough concentration of 1000 ng/l or greater had a full molecular response compared to those with a plasma trough concentration of less than 1000 ng/l ($P<0.05$) [18]. The results of our investigation are consistent with those of the aforementioned studies and suggest that a poor response to treatment may be caused by lower imatinib plasma trough levels [15, 17, 18].

P-gp is a transporter that efflux imatinib from cells, reducing intracellular concentration of imatinib has various consequences [8, 19]. First, imatinib-resistant subclones survive because cells are less likely to go through apoptosis. Imatinib-resistant cell lines produced by cultivating cells at suboptimal concentrations have been found to boost the expression of the BCR-ABL protein. A larger intracellular concentration of imatinib is required to trigger apoptosis at this increased protein level. As a result, leukemic cells become therapeutically resistant [19]. Second, the decrease in intracellular imatinib levels could potentially promote the cells that have mutant copies of the BCR-ABL gene to be selected and grow into larger clones. This would make the protein resistant to imatinib [8, 19]. In addition to leukemic cells, P-gp transporters play an important role in imatinib absorption, distribution, and elimination. The equilibrium between efflux and inflow transport through the intestinal barrier may also have an impact on the imatinib's absolute bioavailability [19].

In our study, the median P-gp expression among responders was 1.12 MFI (range: 1.01-1.38 MFI); P-gp expression was observed in 12 out of 20 patients, or 60%, in the imatinib responder group. 15 out of 20 (75%) patients who were imatinib non-responders exhibited P-gp expression; the median P-gp expression in these patients was 1.16 MFI (range: 1.06-1.50 MFI). There isn't much research that compares the P-gp expression in responders and non-responders of CML-CP patients. Even though studies have examined P-gp expression at various stages of CML, very few have contrasted the P-gp levels in responders and non-responders among CML-CP patients. In a previous study, P-gp expression was observed in 57% of patients in the early chronic phase (at the time of diagnosis) and 65% of patients in the late chronic phase (more than one year from the time of diagnosis) [20]. Vasconcelos et colleagues. found P-gp expression in 89.4% of patients in the early chronic phase [21]. Additionally, although there was a negative connection between P-gp and plasma imatinib trough levels in this study, it was not statistically significant, most likely because of the small sample size. However, despite the lack of statistical significance, this is a significant discovery that should be further examined in large-scale research since it may have implications for the management of imatinib-nonresponder CML patients.

The study has a few limitations that need to be explained. First, there are fewer participants in the research. However, the findings from this pilot study might be expanded upon and a larger study could be

carried out. Additionally, it may be argued that intestinal P-gp was not considered when doing this study; rather, granulocytic P-gp was used as a true representative. However, it has been shown that cellular P-gp expressions can serve as a substitute marker for intestinal P-gp.

CONCLUSION

In conclusion, our study showed that plasma imatinib trough levels correlate strongly with the treatment responders to this medication in CML patients. Furthermore, P-gp plays a crucial role in imatinib response by facilitating the drug's efflux from leucocytes, and P-gp levels are inversely correlated with imatinib levels in the plasma.

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AUTHORS CONTRIBUTIONS

LBN contributed to study design and data interpretation, performed experimental work, and drafted the first version of this manuscript. AVT contributed to data analysis and critical revision of the manuscript.

CONFLICT OF INTERESTS

All authors declare no conflict of interest.

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