UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

MEDICINE

GRADUATE SCHOOL OF

LIFE AND HEALTH SCIENCES

DOCTORAL PROGRAM IN

INFLAMMATION, IMMUNITY AND CANCER

Cycle XXXIII / 2017

TITLE OF THE DOCTORAL THESIS

Successful preservation of BCR-ABL1 protein and direct measure of kinase activity in peripheral blood of CML and Ph^+ ALL patients unveil a kinase inhibitory activity present in chronic phase CML cells.

S.S.D. MED/04

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Tesi di Dottorato
Verona, 10 Febbraio 2021
ISBN

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II. ABSTRACT

BACKGROUND

Chronic myeloid leukemia (CML) is a myeloproliferative disease caused by acquisition of t(9;22) translocation (resulting in Philadelphia chromosome) occurring in a hematopoietic stem cell and transforming it into a leukemic stem cell (LSC) giving rise to a neoplastic clone. The genetic rearrangement lead to the constitutive expression of the fusion tyrosine kinase BCR-ABL1 that alters numerous signal transduction usually governed by growth factors and cytokines. Literature bring back a fast degradative activity present in peripheral blood mature leukocytes, which massively destroys BCR-ABL1 when non-denaturing lysis conditions are applied thus preventing an accurate protein analysis. Data derived from a direct measure of kinase activity in chronic phase (CP) CML are not present in the literature therefore data on the regulation of its enzymatic activity in early phase of the disorder are incomplete.

METHODS

Cell lysis, Immunoprecipitation, Kinase Assay using a phosphopeptide and an anti-phosphotyosine assay (ELISA format). Western blotting with anti-ABL and pABL antibodies.

RESULTS

We aim studying BCR-ABL1 kinase activity in early phase of the disease when data are limited. To achieve our purpose we needed to solve the fast degradation activity present in peripheral blood CML leukocytes and set up a robust kinase assay enabling us to discern specific BCR-ABL1 protein activity. We addressed the first issue creating a CML model obtained mixing healthy donor blood cell with K562 cell line (ratio 10:1) to simulate CML in a peripheral blood context. We then tested several formulations and procedures before lysis and eventually succeeded to preserve BCR-ABL1 protein under non-denaturing, neutral pH conditions. The result was confirmed by western blot and immunoprecipitation techniques. Kinase assay was performed utilizing ELISA format assay based on a biotinylated peptide

substrate highly selective for c-Abl1. We then applied whole procedure on Ph^+ CP-CML and Ph^+ acute lymphoblastic leukemia (ALL) primary cell samples. In this work, I demonstrate for the first time a successful protection of BCR-ABL1 from degradation in the peripheral blood of leukemic patients and the parallel measurement of protein kinase activity in the same samples. Surprisingly we found that tyrosine kinase activity is strongly impaired in CML while activity was readily detectable in Ph^+ ALL-derived leukocytes.

CONCLUSIONS

We have established a new protocol permitting for the first time the direct measure of BCR/ABL1 enzymatic activity in chronic phase CML and applicable in other cellular contexts. Thanks to this original approach we provide the evidence of a novel, still uncharacterized, inhibitory mechanism that maintain BCR-ABL1 in a low active state in chronic phase of chronic myeloid leukemia.

III. INTRODUCTION

Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a biphasic hematopoietic myeloproliferative disorder originated from stem cells (HSCs) but considered progenitor-conduct leukemia[3]. European CML registries report an annual incidence of 0.7-1.0/100,000 with a median age at diagnosis of 57-60 years[4]. The principal hallmark is a single acquired genetic abnormality, translocation t(9;22) (q34;q11) that generate the Philadelphia chromosome (Ph) in hematopoietic stem cell (HSCs)[5,6]. This chromosome is produced by reciprocal translocation between the Abelson leukemia virus oncogene (ABL1) from the long arm of chromosome 9 and the breakpoint cluster region (BCR) from the long arm of chromosome 22 and giving rise to the production of BCR-ABL1 chimeric gene[7]. CML is the quintessentially hematological pathology that harbours the Ph chromosome in more than 95% of cases. Nevertheless, this genetic anomaly is also found in about 10-15% of ALL cases[8]. Research on BCR-ABL1 gene has provided convincing evidence that this early acquisition, occurs in a single HSC, transforming it into the Leukemia Stem Cell (LSC) representing the driving event leading to CML chronic phase[9]. Therefore, aberrant differentiation and/or an increased proliferation capacity over its normal counterparts, allow it to spread (and to be competitive) within the normal myeloid compartment[10].

Three phases in which CML is divided are described according to the guidelines of the European LeukemiaNet[11].

Chronic phase (CP): is defined by the presence of less than 15% blasts in blood[12]. Untreated patients may last in CP for several years in this phase and up to 50% are asymptomatic. Initially, it has a variable duration and time to progression is unpredictable. CP involves an early increase of myeloid cells number in the bloodstream, presenting an immature phenotype but maintaining the differentiation capacity. Patients in CP have a tendency to acquire additional cytogenetic changes in the Ph-positive clone. Therefore, genomic instability is one of the causes of the worsening of the disease[13].

Accelerated phase (AP): is usually a transition phase without any particular symptom changes and hence it can pass unnoticed. AP is characterized by faster expansion of myeloid differentiation-arrested blast cells, usually with a percentage of cells around 15-29% (BCR-ABL1 positive leukocytes in blood or in bone marrow compartment) and which spontaneously evolves into the blast phase. Considering gene expression profiling during CML evolution, lately the disease has been described as a two-step process rather than a three-step process, omitting the AP phase.

Blast crisis (BC): In this late stage, myeloid and/or lymphoid differentiation is blocked and blasts percentage is ≥ 30% in peripheral blood and/or in bone marrow/other tissues. BC lasts for only a few months followed by high mortality rate[7,14]. A plausible hypothesis on BC transformation is represented by multistep and time-dependent processes initiated by both BCR-ABL1–dependent and -independent mechanisms, which leads to cytogenetic abnormalities of other somatic mutations connected with myeloid lineage degeneration[14,15]. Another assumption about CML BC progression is related to the increase in BCR-ABL1 mRNA expression during the disease, and also to an increased risk of being refractory to TKIs treatment[16].

BCR-ABL1 fusion protein architecture

c-Abl1 (Abelson tyrosine kinase; ABL1) is part of non-receptor tyrosine kinases (NRTK) found both in the cytoplasm and nucleus[17]. It is a ubiquitously expressed ~145-kDa protein that in response to numerous stimuli such as cytokines, growth factors, stress factors, DNA damage and other signals is activated by phosphorylation. It interacts with multiple pathways such as proliferation, apoptosis, cell survival and migration[18]. This protein own a tyrosine kinase domain (TKD) mapped upstream of two other SH2 and SH3 (Src Homology 2 and 3) domains having a high sequence homology with the proteins of the SRCs kinase family. c-Abl1 tyrosine kinase activity, being involved in various cellular processes, must be strictly regulated[19]. Both SRC-homology domains together with other segments in the amino-terminal portion, including the CAP domain, generate a

whole of inhibitory intramolecular interactions. Several studies have demonstrated how N-terminal sequences of the SH3 domain bind to the ABL catalytic clef and that they are required together with the N-terminal myristoyl portion for its kinase activity regulation. Specifically, c-Abl shows a myristate moiety ensuring a self-regulatory function; notable, this part is lost upon fusion to Bcr causing the constitutive activation associated with BCR-ABL1. Phospho-tyrosine modification also plays an important role, for example Y245 and Y412 act as kinase activity regulatory sites[20]. Specifically, during c-Abl activation, the phosphorylation on tyrosine Y412 site stabilize the open and active protein conformation. The second regulatory site Y245, located on the SH2-linker, is when phosphorylated prevents the related formation of SH2-SH3 domains regulatory structures. It also promotes Y412 trans-phosphorylation through another c-Abl molecule. Mutations at both sites prevent c-Abl activation[21].

BCR gene encodes a ~160-kDa protein, which is ubiquitously expressed as c-Abl. This protein was originally identified through its involvement in the Philadelphia (Ph) chromosome translocation. Studies have demonstrated that BCR protein may act as a GTPase activating protein (GAP), which play an important role in chemical signalling within cells. It is composed by multiple domains, including a C-terminal GTPase-activating domain, N-terminal residues that contains a region with serine/threonine kinase activity and a Coiled-coil domain (tetramerization domain)[22]. Certain study models highlight the oncogenic protein oligomerization through the Bcr coiled-coil domain, leading to mutual trans-phosphorylation of close c-Abl kinase domains in BCR-ABL1, and thus originates a constitutively active tyrosine kinase[23,24]. BCR-ABL1 leukemogenic potential is remarkably reduced by mutations of the coiled-coil domain caused by auto-phosphorylation impairment in regulatory sites[23].

BCR-ABL1 hybrid gene is generated by juxtaposing the 3'-end of ABL1 strand gene a 5'-end of a strand part of the BCR gene (breakpoint cluster region). In particular, ABL1 gene has several possible breakpoints with a higher frequency within the gene region between exons 1a and 1b. Likewise, inside the BCR gene is possible to consider three cluster regions (Major, minor and μ), which can produce at least three different fusion genes, each coding proteins distinguished by different

molecular weight[8]. Different BCR-ABL1 forms appear to be related to particular leukemic phenotypes. Indeed, the majority of CML patients and in one-third of Ph^+ B-ALL the BCR break points are localized within the Major cluster region (M-bcr) which is involved in the production of $p210^{BCR\text{-}ABL1}$ (transcripts of function b2a2 or b3a2). On the other hand, minor cluster region (m-bcr) included between exons e2′ and e2 generates $p190^{BCR\text{-}ABL1}$ accounted inside the other two-thirds of B Acute Lymphocytic Leukemia (**Figure 1**). The third protein form, involving the µ-bcr region, generates a heavier fusion protein $p230^{BCR\text{-}ABL1}$ present in chronic neutrophilic leukemia (CNL)[7,25] but it is also detected in CML and AML[26]. Some sporadic CML cases have been associated with the $p190^{BCR\text{-}ABL1}$ form, although this is mostly associated with lymphocytic leukemia. These two infrequent forms are often associated with a worse response to therapy[27].

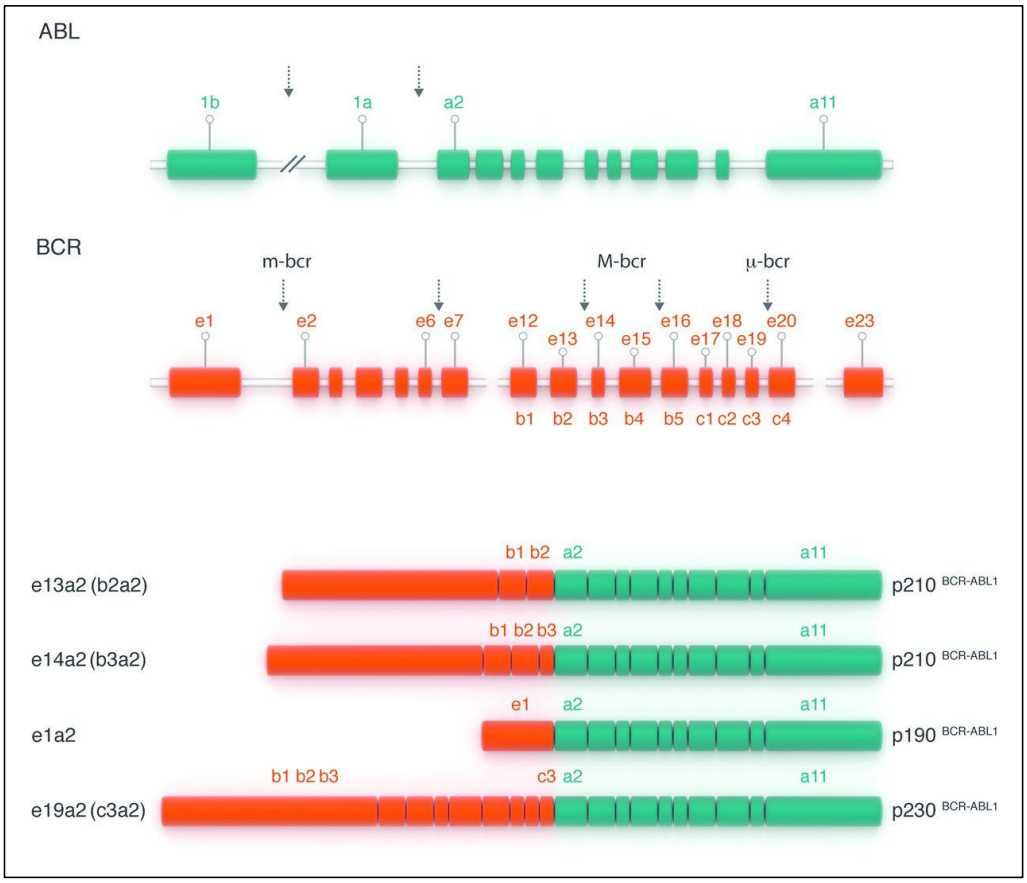


FIGURE 1 Sequences representation of BCR and ABL1 normal genes and possible BCR–ABL1 mRNA transcripts found in Ph-positive Leukemias. ABL1 gene consists of two alternative exons 1b and 1a where is located the breakpoints site and in the BCR gene are shown the three cluster regions.[1]

Overview of known dysregulated mechanisms driven by BCR-ABL1

The structural chromosomal rearrangement caused by BCR and ABL juxtaposition involves the loss of all regulatory mechanisms that characterize their respective physiological proteins. BCR-ABL1 can affect many substrates, including kinases, altering numerous intracellular signal cascades. The phosphorylated Y177 site found in BCR moiety plays a crucial role in the disease, indeed point mutations on tyrosine, like Y177F, widely suppresses the oncoprotein leukemogenic potential. Y177 operates as a high-affinity docking site for the SH2 domain of GRB2, which binds scaffold adapter GRB2-associated binding protein 2 (GAB2), promoting GRB2/GAB2 protein engagement[28]. The complex formed is activated by BCR-ABL1-induced phosphorylation, resulting in PI-3K/AKT dysregulation[29]. Simultaneously, GRB2 binds SOS (a guanine-nucleotide exchanger of RAS), producing RAS activation thereby promoting mitogen-activated protein (MAP) extracellular signal-regulated kinase (ERK 1/2), (MEK) and MAP kinase proteins and in the end resulting in abnormal cell proliferation. CML cells use BCR-ABL1-induced AKT activation to regulate apoptotic factors, such as BAD, thus negatively modulating apoptosis[30]. On the other hand, RAS-recruited and activated by the Grb2-SOS complex is able to modulate several Bcl-2 family proteins involved in the mitochondrial caspase cascade[31]. Therefore, downstream-dysregulated PI3K/AKT and RAS pathway in BCR-ABL1 cells provides some of the apoptosis-evasion mechanisms in leukemia cells[32].

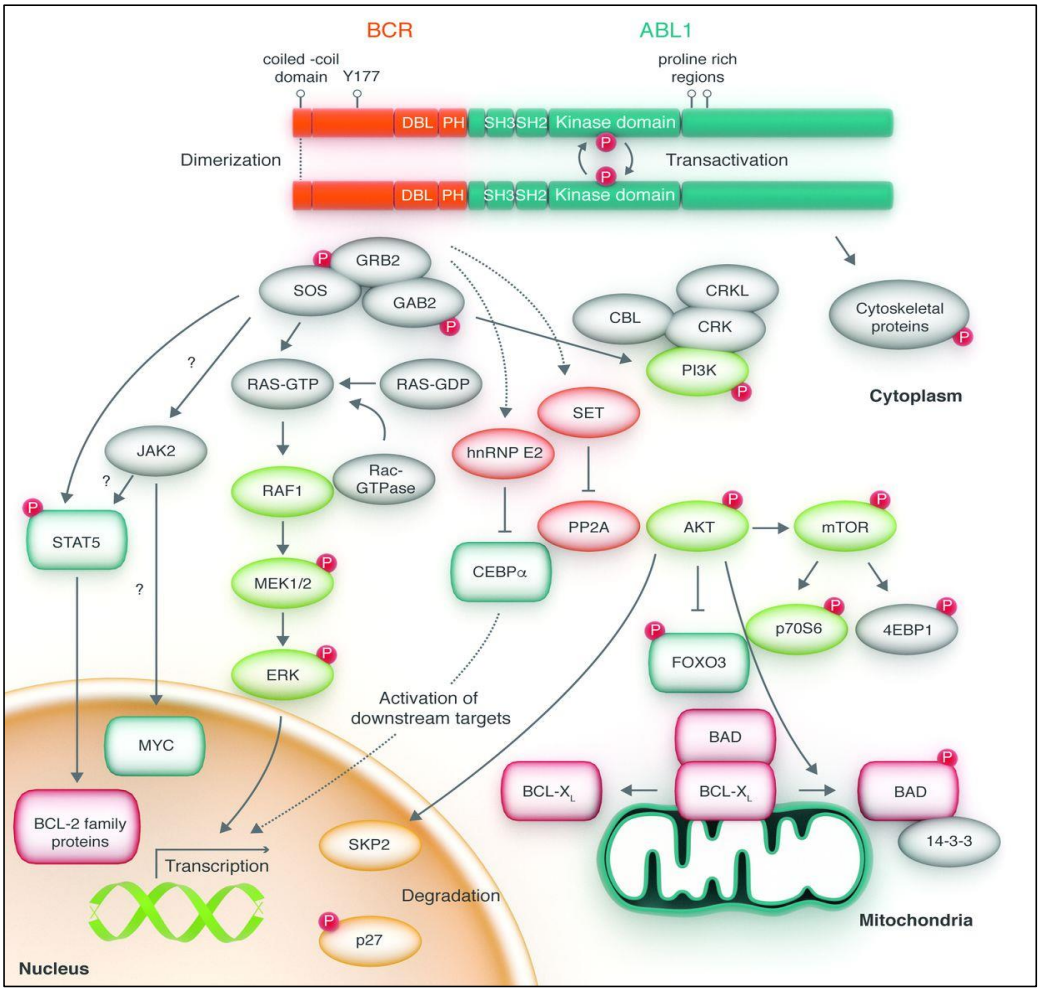


FIGURE 2 BCR-ABL1 protein structure and schematic representation of molecular signalling pathways deregulated in chronic myeloid leukemia (CML) cells[1].

Usually ABL1 shuttles between the nucleus and the cytoplasm; on the contrary, its oncogenic form focuses its activities entirely in the cytoplasm, where it can phosphorylate and affect the majority of proteins involved in the leukemia pathway. It is worth mentioning that several key transcription factors are involved in BCR-ABL1 signalling. Especially, STATs (signal transducer and activation of transcription) protein family is widely recruited, contributing to advantages in Ph⁺ cells vitality. STAT5 is partially responsible for programmed cell death protection affecting anti-apoptotic Bcl-2 and Bcl-XL genes transcription. STATs transcription factors are normally related to the JAKs family kinases, which have the function of signal transmitter, attached to external biochemical stimuli, such as cytokines (IL-

3, GM-CSF and IL-6). Instead, in CML, STAT proteins are activated in a JAK-dependent manner and can be also phosphorylated directly by BCR-ABL1, bypassing JAK family kinase activation[33]. These evidences confirm the mutual-interaction among BCR-ABL1-JAKs family network[34]. Notably, BCR-ABL1-expression is indeed sufficient to recruit and activate JAK2/β-catenin cascade, involving another key transcription factors. Ph-positive cells increase their cytoplasmic β-catenin levels compared to normal HSCs, affecting self-renewal and survival signals[35]. β-catenin was shown to act also through Sonic-hedgehog pathway in CML progenitor's cells[36]. To further sharpen it is possible to mention that an additional important mediator is represented by stromal niche, where various factors are deregulated such as CXCR4 and TGF-β, increased ROS (reactive oxygen species) production[32], mitotic process alteration (AURORA Kinase) and loss of tumor suppressors function (PP2A, PTPRG)[35]. Surprisingly a wealth of data confirm the evidence of LSC ability to be dispensable from the BCR-ABL1 kinase activity in CML, which can be boosted with the disease progression[37].

The power of targeted therapy in Chronic Myeloid Leukemia

Several experimental approaches, such as CML CD34+ cells cultures or retrovirally transduced BCR-ABL1-positive mouse cells[38], have established that BCR-ABL1 protein is central to the pathogenesis of CML, which has offered the rationale for the development of tyrosine kinase inhibitors (TKIs) for CML treatment. Initially, the first advance to improve CML patient survival was based on a ribonucleotide reductase inhibitor, Hydroxiurea[39]. Thereafter the treatment evolved with IFNα use, which resulted in an effective cytogenetic response. Over the last two decades, Tyrosine Kinase Inhibitors (TKIs) have been a major innovation in the treatment of dysregulated kinases. Imatinib Mesylate (STI571; Glivec©) was approved in 2001 by FDA and it is nowadays used as the standard treatment in newly diagnosed CP-CML patients. The antineoplastic agent binds the BCR-ABL1 TK-domain occluding the ATP-binding cleft and stabilizing the protein in an inactive state. The inhibitor binding blocks the BCR-ABL1 auto-phosphorylation capacity, which is

essential to supply the disease cellular processes, resulting in a reduction of leukemic burden associated to an increasing apoptosis[41]. Based on the assumption to achieve a better response to TKIs therapy through combination of drugs, several clinical trials have been established assuming a cumulative effect with IFNα plus TKIs. However, the potential use of IFNα as combination therapy is somewhat limited by its incumbent toxicity[40].

Afterward the use of Imatinib as first-line therapy (standard daily-dose 400 mg for CP) demonstrate rapid achievement of durable remission and MMR (Major Molecular Response) rates range between 20–59% at one year and 60–80% at 5 years. Furthermore, five-years PFS (Progression Free-Survival) and OS (Overall Survival) ranges have been described being between 80–90% and 90–95% respectively, with an about 6% leukemia-related death rated[42].

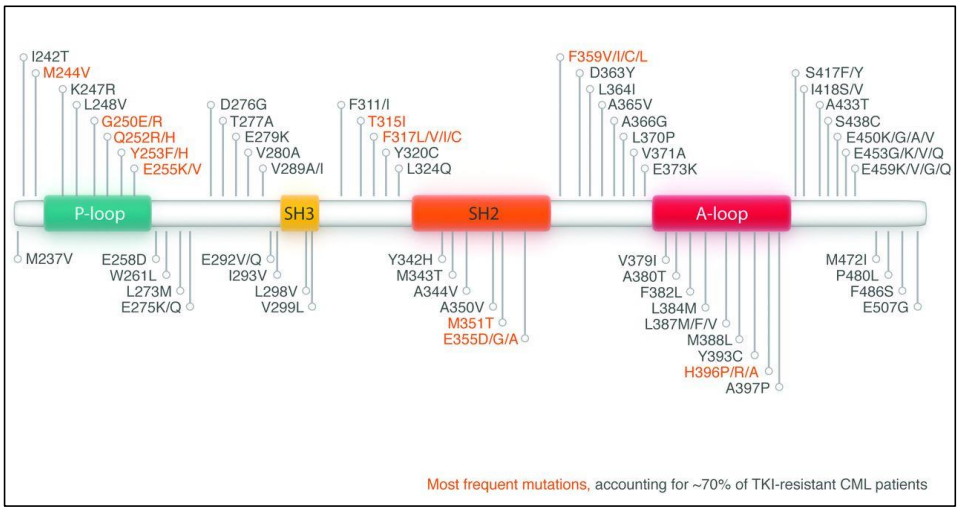


FIGURE 3 Possible site mutations in ABL1 kinase domain detected in drug resistant CML patients[1].

However, despite the relative excellent results obtained by the Imatinib treatment, approximately 20–30 % of CML treated-patients will develop resistance to therapy. Different cellular and molecular processes, such as drug influx/efflux pumps downregulation and BCR-ABL1 over-expression, have been described as CML drug-resistance phenomena. Conceivably, the most common mechanisms remain kinase domain mutations (**Figure 3**), following replacements of amino acids in the ABL-protein structure, producing TKIs-binding alteration. These alterations reduce

or eliminate drug effectiveness without inducing a normalization of haematological values.[32] To overcome mutation-dependent IM resistance and for those patients who fail therapy due to intolerance, a set of more selective FDA-approved second-generation TKIs are available, such as Nilotinib[43] which is a more potent c-Abl kinase inhibitor and Bosutinib or Dasatinib[44] both dual-kinase inhibitor of c-Abl and Src kinases[42] (**Figure 4**).

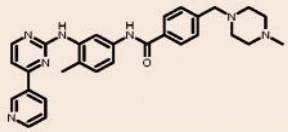
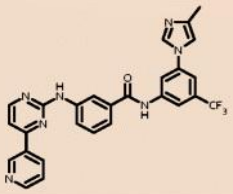
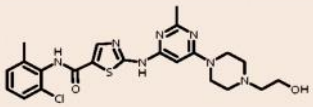
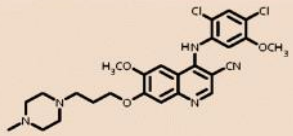
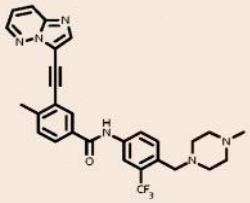
Inhibitor	Chemical structure	Binding site/ Inhibitor type	Regulatory status/ approval
Imatinib (Gleevec)		ATP-binding site/ ATP-competitive	FDA approved/ frontline therapy
Nilotinib (Tasigna)		ATP-binding site/ ATP-competitive	FDA approved/ frontline therapy
Dasatinib (Sprycel)		ATP-binding site/ ATP-competitive	FDA approved/ frontline therapy
Bosutinib (Bosulif)		ATP-binding site/ ATP-competitive	FDA approved/ 2nd-line therapy
Ponatinib (Iclusig)		ATP-binding site/ ATP-competitive	FDA approved/ 2nd-line therapy
ABL001	(Currently proprietary)	Myristate pocket/ allosteric	Phase I/ 2nd-line therapy

FIGURE 4 Molecular structures of FDA approved tyrosine kinase inhibitors including the promising ABL001 (Asciminib).

Pharmacologically, the most problematic alteration is the T315I BCR-ABL1 'gatekeeper' mutation, characterized by a fundamental hydrogen binding deprivation in the ATP pocket, involved in the high-binding affinity with the four aforementioned drugs. Ponatinib, a third generation TKI is a kinase inhibitor optimized to bind the ABL inactive conformation and its T315I mutation[45]. To further overpass TKD-mutation resistance, small allosteric inhibitory molecules have been developed outside the kinase domain (e.g. Asciminib - ABL001) that are able to bind the myristoyl pocket mimicking the native inhibition mechanism[46]. These data suggest the requirement to treat Ph-positive leukemia during chronic-phase (CP), thereby reducing prematurely the disease burden and reach TKIs-durable responses with disease-free remissions event[47]. Most often, the TKIs-medication must be continued indefinitely and therefore the overriding challenge now remains to investigate current FDA-approved drugs with the possibility to develop new safe and effective therapy-strategies.

Leukemia diagnosis and monitoring.

Conventionally, identification of the Philadelphia Chromosome is carried out by classical cytogenetic techniques with chromosome banding (CBA) screening of 20 marrow cell metaphases. Alongside, FISH (Fluorescent In Situ Hybridization) with higher sensitivity is also exploited through genomic fluorescent probes, which are able to recognize the fusion gene. Hematological and karyotype monitoring allow blood parameters surveillance and identification of possible chromosomal abnormalities to achieve complete hematologic and cytogenetic response (CHR and CCyR) during treatment. The progress of molecular biology has made possible to go beyond CHR and CCyR and to control residual disease on a molecular level[48]. These analysis strategies are supported by RT-PCR, a suitable technique for a precise quantification of BCR-ABL1 transcript (mRNA). Interestingly, an early reduction of BCR-ABL1 transcript has been shown to represent a powerful prognostic factor, predicting cytogenetic response and clinical outcome in treated patients[49]. More precisely, lack or slight TKIs-induced reduction in the fusion gene transcription must be a warning sign. Guidelines recommended by ELN (European

LeukemiaNet) and NCCN (National Comprehensive Cancer Network) underline importance of the disease monitoring 3 - 6 months after the start of TKI therapy, in order to identify any pharmacological failures or intolerance and to optimize patient-therapies with a low response[11]. For molecular monitoring, PCR-based tests that detect and quantify BCR-ABL1 mRNA are considered the "gold standard" methods since they show a high sensitivity (detecting as few as 0.001% of positive cells).

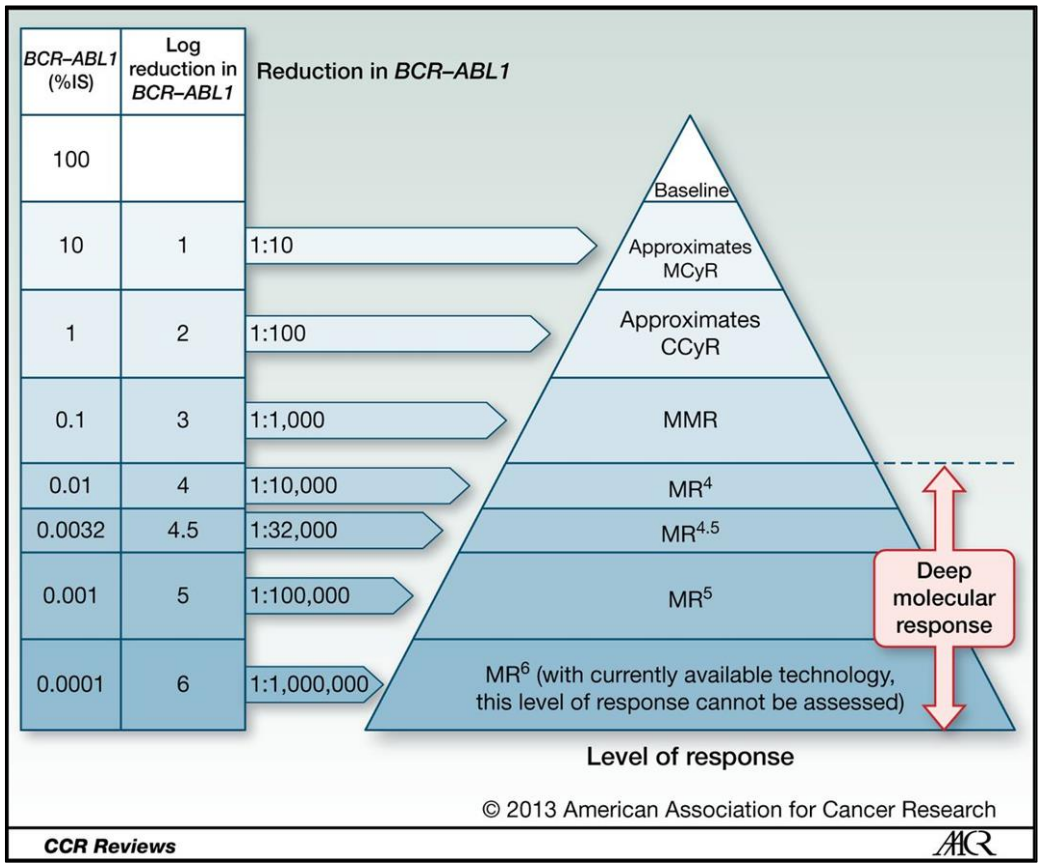


FIGURE 5 Levels of molecular response in CML. MCyR, major cytogenetic response; MR, molecular response.[2]

Molecular response is assessed as BCR-ABL1 ratio transcripts to ABL1 transcripts, (or other internationally recognized control transcripts [BCR or GUSB]. It is expressed and reported as BCR-ABL1 log value, where decreasing percentage

values of BCR-ABL1 transcript correspond to a decrease of 1, 2, 3, 4, 4.5, and 5 logs (**Figure 5**) according to the International Scale (IS). The major molecular response (MMR), which definition arises from the IRIS study[50], outlines the first level of response evaluated on the molecular scale and it corresponds to a 3-log reduction in BCR-ABL1 transcript levels from a standardized baseline (≤0.1% BCR–ABL1 on the IS). This amount of residual disease is significant in predicting the risk of disease progression for newly diagnosed CML patients, thus achieving a complete cytogenetic response (**Figure 5**).

Achievement of MMR is still a reference during TKIs treatment. Progress in standardization of the RQ-PCR technique has witnessed the understanding of drug response course through the Deep Molecular Response (DMR) effectiveness. DMR is usually explained as BCR-ABL1 values under ≤ 0.01% IS threshold and it is described as various BCR-ABL1 cut-off values. Basically molecular response 4 (MR4) is ≤ 0.01% IS, MR4.5 ≤0.0032% IS, and MR5 is <0.001%[51]. CML patients who reach deep molecular response seem less likely to lose MMR, demonstrating how DMR is related with better long-term clinical outcomes, such as EFS (Event Free Survival), PFS (Progression Free Survival), and OS (Overall survival), and a low risk of progression and disease relapse. Hence, the timely clinical goals in CML will be the amelioration of chronic low-grade side effects and reach a deep response, which then will allow patients to reach successful TKI discontinuation. The increased chances to achieve a treatment-free disease (TFR) control will enhance the quality of life in these patients.

Alternative approaches for therapeutic prediction and disease evolution.

Notwithstanding consistent standardization processes regarding RT-PCR technique in the monitoring patients undergoing TKIs, the risk of therapy failure owing to mutations or intolerances, is still considered a barrier of great study interest. Re-evaluating BCR-ABL1 protein significance, in addition to its transcript measurements, has been attempted in the last two decades and different approaches have been proposed as new prognostic tools. BCR-ABL1 protein recognition

through flow-based assays has also been shown as alternative way to evaluate CML cases.[52] Several approaches have demonstrated the binding of fusion proteins to immune-beads covered with antibodies suitable to identifying a specific protein moiety. Simultaneously, another fluorescence-labelled antibody binds specifically to a different BCR-ABL1 protein portion. Finally, beads are evaluated using a flow cytometer, which can point out the immuno-complex sandwich containing the fusion protein[53,54]. Studies of cellular phosphorylation events have acquired greater importance considering the key role of kinases in various cellular processes such as growth, differentiation, etc. This relevance is capitalized in aid of the pharmaceutical industry, which have had the strength to develop and design new enzyme inhibitor drugs being useful in numerous pathologies greatly thanks to this kind of screening tests[55]. Therefore, it might be important to be able to directly assay BCR-ABL1 kinase activity as it might be a potential parameter to predict response and supervise the disease in a condition as close as possible to the patient. Consequently, multiple *in-vivo* BCR-ABL1 kinase activation state measurements have been conceived studying kinase substrates such as pCRKL protein[56], which is described as a constitutively tyrosyl-phosphoprotein detected in mature CML cells. Quantification of CRKL-phosphorylation (although being an indirect indicator of BCR/ABL1 activity) with proteomics or with flow cytometric devices, may supply an excellent predictor of MMR and may be a precious guide to therapy adjustment[57-59]. Meanwhile a cell-based assay for directly measuring endogenous BCR-ABL1 activity has been proposed in many human Ph^+ cell-lines[60]. This biologically relevant kinase assay provides also a practical test to measure efficiently small inhibitory molecule[61]. However, these tests have never been validated in primary leukemic samples. The explanation seems to be related to a long and lively debate in literature, regarding BCR-ABL1 protein expression in primary CML cells, which apparently vanishes owing to a strong enzymatic-specific degradation[62,63]. Historically, the first successful attempt to identify the protein in primary CML leukocytes is a protein denaturing procedure described by Guo *et al* required high temperature (i.e. 95°C) denaturing lysis buffer to inhibit degradative activity in primary leukemia cells[64]. Mature CML granulocytes have extremely high levels of proteases in their granules in contrast to their CD34+

precursor cells that lack significant protease activity[62]. Focused on this point, Patel's group described a specific protease activity, inhibited by high pH, whereby NaOH was added in lysis buffer (final concentration 0.125 to 1M) resulting in a complete block of the action of acid-dependent hydrolases being released during cell lysis[65]. Another kit distributed by Becton-Dickinson[54] was apparently able to halt protein degradation through AEBSF, PMSF and ZAP-OGLOBIN reagent included within the lysis system. Doubts regarding the antigens bands detected and batch-to-batch variation efficacy of the ZAP-OGLOBIN reagent, together have now lead to product discontinuation. All these aforementioned lysis conditions preclude the study of BCR-ABL1 kinase activity derived from primary cells, because the protein-denatured state is incompatible with activity-based assays where preservation of the native conformation is essential.

IV. AIMS OF THE STUDY

The steady awareness increasing about CML disease makes the quality and life expectancy of CML patients acceptable. This awareness has, for instance, lead to improvement and standardization of diagnostic and monitoring methods but also the relentless development of kinase inhibitor drugs (TKIs). However, despite this comforting situation, the complexity of the pathological phenomenon allows few patients to achieve DMR leading to subsequent discontinuation of the therapy. Therefore, a better understanding of the mechanisms of BCR-ABL1 protein regulation will allow to build a more detailed picture of this disease. The direct BCR-ABL1 activity measurement from primary CML-samples remains unexplored owing to the strong degradation activity of the target occurring in primary leukocytes as discussed. Overcoming this limitation would open the possibility to study regulation of kinase activity in newly diagnosed chronic phase CML. This would further increase the understanding and evaluation of the role that this kinase plays in the disease, other than opening new scenarios on the feasibility of a specific kinase assay for rapid prediction to the response of patients to TKIs. In order to reach these objectives this project was focused on:

- Set up of a reliable assay measuring enzymatic activity of BCR-ABL1 protein in Ph^+ cells lines samples using an ELISA-based kinase assay.
- Set-up of a non-denaturing lysis procedure for BCR-ABL1 protein preservation in order to retain the native protein folding and its enzymatic activity from CP-CML primary cells (Leukoprotect procedure).
- Quantitative evaluation of the kinase activity derived from Ph^+ CP-CML and ALL.

V. MATERIALS AND METHODS

Cells lines

Murine pro-B cell line Ba/F3 cells were maintained in in RPMI 1640 (Life Technologies, Inc., Gaithersburg) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2mM L-glutamine, 10% conditioned medium from WEHI cell culture producing murine recombinant IL-3 and incubated at 37°C with 5% CO_2 atmosphere. K562, LAMA84, JURKAT and MM6 cells lines were cultured as above without addition of murine IL-3. All cell lines were routinely tested for mycoplasma contamination.

Kinase inhibitors

Imatinib (STI-571) and Dasatinib (BMS-354825) were purchased from Selleckchem (Houston, TX). Stock solutions of Imatinib and Dasatinib at 1 or 10 mM in sterile water were filtered and stored at −20°C. For Imatinib (1μM) and Dasatinib (1nM) exposure, the indicated concentration was added to cell cultures for the indicated times before harvesting the cells for Western blot analysis and immunoprecipitation (IP) experiments.

Patient samples

Patients in this study were enrolled onto the trial "**S**tudio sperimentale esplorativo sul monitoraggio **PR**ecoce mediante biosensore dell'attività degli **IN**ibitori delle **T**irosin chinasi in pazienti con leucemia mieloide cronica (SPRINT)" a study conducted on adult patients with newly diagnosed CML. EDTA-anticoagulated peripheral blood (PB) cells were obtained from newly diagnosed patients with CP CML. All patients were enrolled within 7 days of diagnosis, without history of TKIs or interferon therapy.

Thawing procedure for patient samples

ALL Ph^+ samples were kindly provided by Prof. Bonifacio (Hematology section of the Department of Medicine,Verona). Briefly, cells were thawed in IMDM (Iscove Modified Dulbecco Media) at 37 °C containing 10 U/mL of Deoxyribonuclease I (Sigma - St. Louis, MI) to avoid clumping. Cells were centrifuged at 200xg for 15 minutes and then treated according to the Leukoprotect protocol, as described below.

Cell Lysis and LeukoProtect buffer:

Whole blood is suspended in LeukoProtect pretreatment buffer: Roche tablet - cOmplete™ ULTRA Tablets, Mini, EDTA-free (Sigma - St. Louis, MI) were resuspended in PBS then solution A, solution B and 10mM EDTA were added. Solution A and B are protected by industrial secrecy agreement with AB Analitica, Padova. After 20 min of incubation at room temperature (in continuous slowly shaking), red blood cells (RBC) osmotic lysis was performed (RBC lysis buffer: 0.155M NH_4Cl, 0.01M $KHCO_3$, 0.1mM EDTA in H_2O). Cells were centrifuged at 200xg for 5 minutes, supernatant was removed and the pelleted leukocytes were lysed in an appropriate volume of cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 2 mM EDTA and protein inhibitor cocktail in H_2O). Samples were kept on ice for 15 minutes and centrifuge at 13,000 rpm at 4°C for 15 minutes. Protein concentration of cleared lysate was quantified using the Bradford protein assay (SERVA Electrophoresis, Heidelberg, Germany). Lysate aliquots were stored at -80°C.

BCR-ABL1 Immunoprecipitation:

Protein lysates were incubated with an antibody against total c-Abl (Cell-Signaling Technology, Danvers, MA) for 2 hours. Protein G-Dynabeads (Invitrogen, Carlsbad, CA, USA) were added and the mixture was incubated for 1 hour. Both steps were performed at 4°C on a rotating wheel. Kinase assay on ABLtide peptide substrate was performed and beads containing BCR-ABL1 protein were than

denatured in Laemmli buffer for 5' at 95°C before separation on SDS-polyacrylamide gels.

Western blotting

Protein samples in SDS–PAGE gels were transferred onto PVDF membranes (Bio-Rad, Hercules CA). Blocking was performed using a solution of PBS 0.1% Tween 20/BSA 3%. Incubations with primary antibodies were done overnight at 4°C in a shaking system. Incubation with secondary antibodies were performed for 1 hour and horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG (Cell-Signalling Technology, Danvers, MA) antibodies were used. All antibodies employed according to manufacturer's recommendations, and they are indicated in **Table 1**. Antibodies were detected using the enhanced chemiluminescence with ECL Supernova (Cyagen, Santa Clara, CA). All images were acquired with the digital imaging system ImageQuant®LAS 4000 biomolecular imager (GE Healthcare Europe GmbH, Germany). The bands were quantified using the ImageJ software to obtain optical densities values, which were then normalized with indicate housekeeping proteins.

Optical density detection

Densitometry values of blotted bands were quantified using the ImageJ software and then normalized with indicate housekeeping proteins. Meanwhile, OD values for BCRABL1 immuno-precipitated from primary samples were obtained by threshold analysis and using a calibration curve, derived from different amount of BCR-ABL1 immuno-precipitated from K562, were extracted through ImageJ software in order to quantify the western blots (**Figure 6**).

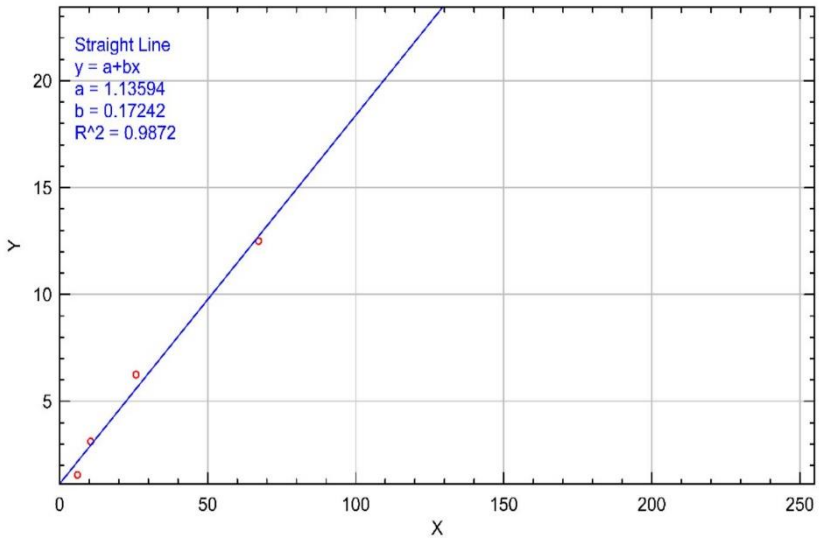


FIGURE 6 Calibration curve obtained from immuno-precipitated K562 lysates. Y-axis represent the values (1.56 - 3.12 - 6.25 - 12.5) in µg of K562 lysate from which BCR-ABL1 was immuno-precipitated, while on the X-axis the related densitometry values.

Kinase assay

We utilized a peptide designed and validated as specific for ABL kinase (Sequence: EAIYAAPFAKK-biotinK-GGCGGAPTYSPPPPPG)[60,66,67]. After BCR-ABL1 immunoprecipitation, but before denaturation in Laemmli Sample buffer, 1µM ABLtide (GenScript Biotech, Piscataway, NJ) resuspended in Kinase Buffer (20mM Tris-HCl, 10mM $MgCl_2$, 0.05% Triton-X, 20µM ATP, 1mM DTT, 0.1mM Na_3VO_4 in H_2O) was added to the immunocomplexes for 30 min on a rotating wheel at 4°C. The biotinylated peptide was captured on NeutrAvidin™ Coated High Capacity Plates (Thermo Fisher Scientific - Waltham, MA). Next, wells were washed with PBS Tween20 0.1% pH 7.4 and, primary mouse monoclonal anti-phosphotyrosine antibody, clone 4G10 (Millipore Corporation, Bedford, MA) was added and then incubated at 4°C overnight. Plates were washed and incubated with anti-mouse IgG antibody conjugated with HRP for 1h at room temperature. Lastly, developing solution Amplex Ultra Red Reagent (Invitrogen, Carlsbad, CA) was added. Plates were analysed in Victor plate reader (PerkinElmer - Waltham, MA)

at 540 nm excitation, 572 nm emission wavelengths. Finally, the slope analysis was performed.

ANTIBODY	CODE	HOST	COMPANY
Phospho-c-Abl (Tyr245)	#2861	Rabbit	Cell Signaling
c-Abl	#2862	Rabbit	Cell Signaling
Jak2 (D2E12)	#3230	Rabbit	Cell Signaling
Syk (D3Z1E)	#13198	Rabbit	Cell Signaling
Btk	Ab54129	Mouse	Abcam
Akt	#9272	Rabbit	Cell Signaling
Lyn	Sc-15 (44)	Rabbit	Santa Cruz
Sapk/Jnk	#9252	Rabbit	Cell Signaling
Erk 1/2	#9102S	Rabbit	Cell Signaling
Mapk p38	#9212	Rabbit	Cell Signaling
Anti-Phospho 4G10 clone	05-321	Mouse	Merk-Millipore
β-Actin (13E5)	#4970	Rabbit	Cell Signaling

TABLE 1 Antibodies table that summarize those used in this study.

Statistical analysis

Analysis were performed using GraphPad 7 Instat software and data are displayed as the mean ± S.D. P-values were calculated using Student's t-test for evaluation of significant difference between groups. The definition of statistically significant P-values was indicated directly on figures.

VI. RESULTS

BCR-ABL1 activity detection in different human leukemia cell lines

Based on the pre-existing knowledge about kinase assays in our laboratory, we have developed an immunoprecipitation step in order to eliminate non-specific background that could interfere with the specificity of the signal associated to the reporter peptide. Therefore, we first analysed the assay efficiency by measuring BCR-ABL1 enzymatic activity. In particular, we started from lysates derived from four human cell lines with leukemic phenotypes that two carring the *BCRABL1* fusion gene (K562 and LAMA84) while the other two as negative controls (Jurkat and MM6).

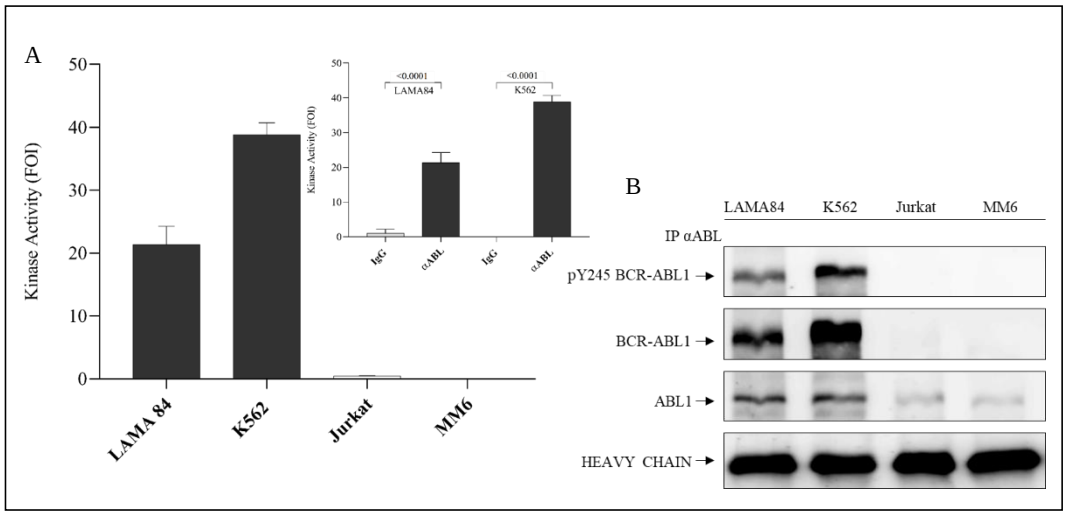


FIGURE 7 BCR-ABL1 kinase activity in different leukemic cell lines.

A) Kinase activity recorded by immunoprecipitation performed using a specific antibody against ABL1 (#2862 Cell-Signaling Technology) starting from equal lysates derived from four leukemic cell lines, Ph^+ positive K562 and LAMA84 cell lines are compared with Ph^- Jurkat and MM6. Corner bar graph represents the same kinase activity experiment performed exclusively on Ph^+ lines using both a rabbit IgG compared with ABL1. No signal is detectable in control immune-precipitates (Paired t-test was applied for both cell lines with a significant p value (<0.05). **B)** Immuno-precipitates from panel A run on SDS-Page gels and subjected to western blotting with anti-ABL1 and anti pY245-ABL1.

Bar graph in Figure 7A shows the activity recorded in four cell lines, highlighting a noticeable signal only in the two Ph-positive cell lines LAMA84 and K562. The results demonstrate the ability of the assay to detect the activity of the BCR-ABL1 protein owing to its strong dysregulation[23]. Analysis of Ph-negative cell lines and the rabbit IgG-controls (**Figure 7A**) did not register a tyrosine-phosphorylation of the reporter peptide suggesting that: 1) signal is associated with enzymatically active BCR-ABL1, while ABL enzymatic activity is downregulated in resting conditions (Jurkat and MM6 cell lines). 2) The immunoprecipitation procedure specifically captures BCR-ABL1 and ABL1, as no any signal is detected when rabbit IgG control is used for immunoprecipitation (**Figure 7A**).

Western blot of the immune-precipitates run on SDS-page showed the correlation between activity and BCR-ABL1 protein expression in LAMA84 and K562 (**Figure 7B**). On the other hand, two Ph-negative cell lines exhibits only ABL1 protein, whose enzymatic activity is known to be strongly downregulated and activated only upon post-cellular stimulation (such as DNA damage)[18]. As expected, it does not produce detectable enzymatic activity (**Figure 7A**).

Validation of the kinase assay

Having established the procedure to recognise BCR-ABL1 enzymatic function, we moved on analysing the reproducibility and sensitivity of the assay. We cultured both CML cell lines (K562 and LAMA84 respectively) for 3 hours with 5µM Imatinib (TKI) and after lysis step kinase activity assay was performed. Each scatter plot (**Figure 8A and B**) describes either “untreated condition” or "Imatinib-treatment condition" in which the drug inhibits the phosphorylation of the specific reported substrate. The kinase assay in both CML cell lines was repeated several times to demonstrate assay repeatability (student t-test: $p < 0.001$ for K562 and $p =$

0.012 for LAMA84). We then tested kinase assay sensitivity by keeping constant all immunoprecipitation conditions whereas we varied concentrations of protein lysate originating from a positive BCR-ABL1 cell line (K562). As expected, there is a linear correlation between kinase activity and amount of protein lysate that remain detectable up to as little as a few micrograms of total cell lysate (**Figure 8C**

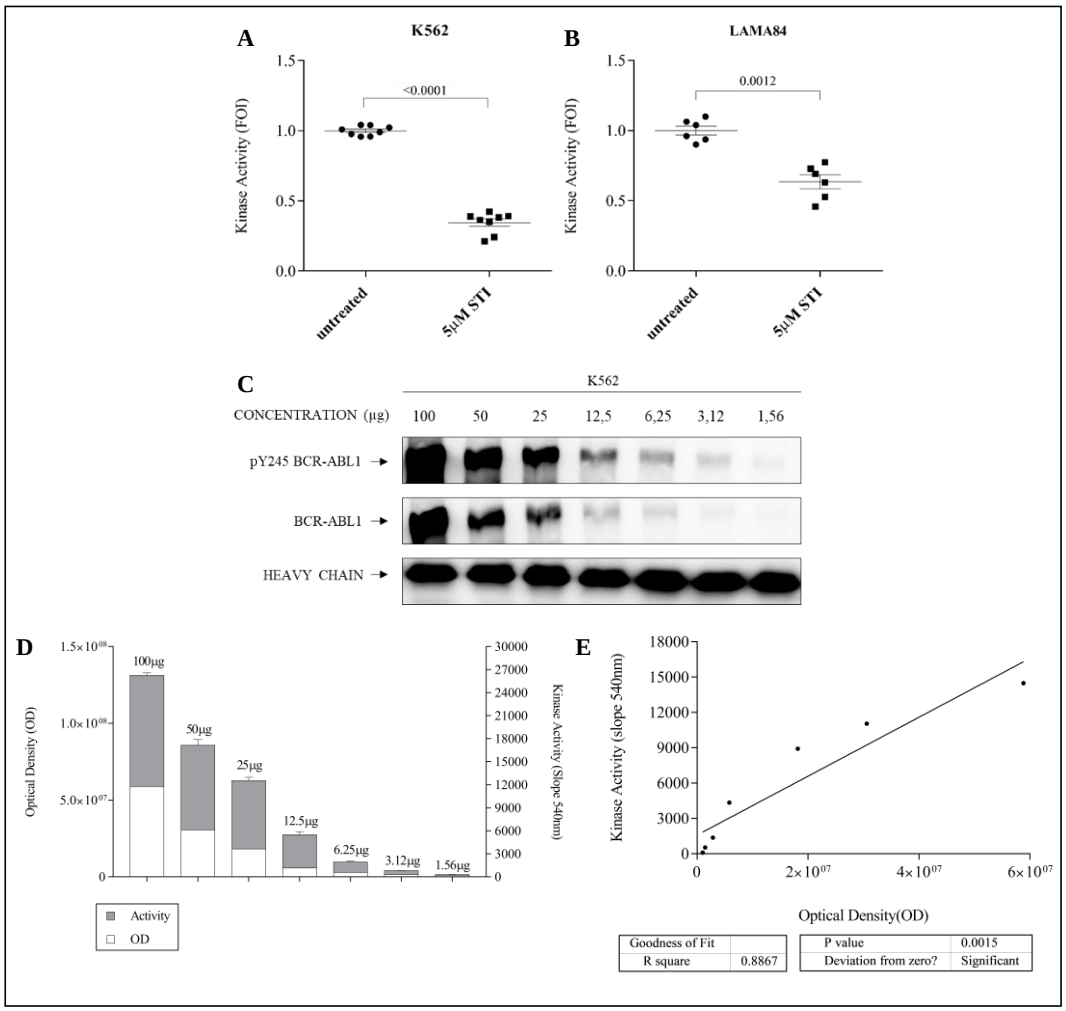


FIGURE 8 Peptide biosensor-based assay by repeatability and sensitivity validation.

A and B) Each CML cell lines were cultured for 3 hours with 5µM IMATINIB (STI) lysed and immuno-precipitated with anti ABL1 antibody (#2862 Cell-Signaling Technology) before kinase assay. Procedure was repeated n= 8 for the K562 and n= 6 for LAMA84. Paired t-test was applied for both cell lines with a significant p value (<0.05). **C)** Western blot analysis on immuno-precipitated samples**. D)** Kinase activity (grey) and OD-optical density (white) value expressed as densitometry analysis of immuno-precipitated BCR-ABL1 of CML cell line K562. The amount of starting lysate was indicated above each bar. **E)** Pearson correlation analysis of kinase activity plotted versus OD values of the graph shown in figure 7D. Linear regression analysis: R^2 and P value of the regression were shown below the graph.

and D), also showing an optimal Pearson Correlation Coefficient $R^2 = 0.9417$; P = 0,0015 (**Figure 8E)**.

There are different modalities of how TKI-resistance can originate in Chronic Myeloid Leukemia, but the primary mechanism remains the acquisition of gene sequence mutations leading to a protein sequence modification (usually in tyrosine kinase domain-TKD). This modification makes the drug-protein binding weaker or non-existent, leading to a decreased TKIs efficacy[68].

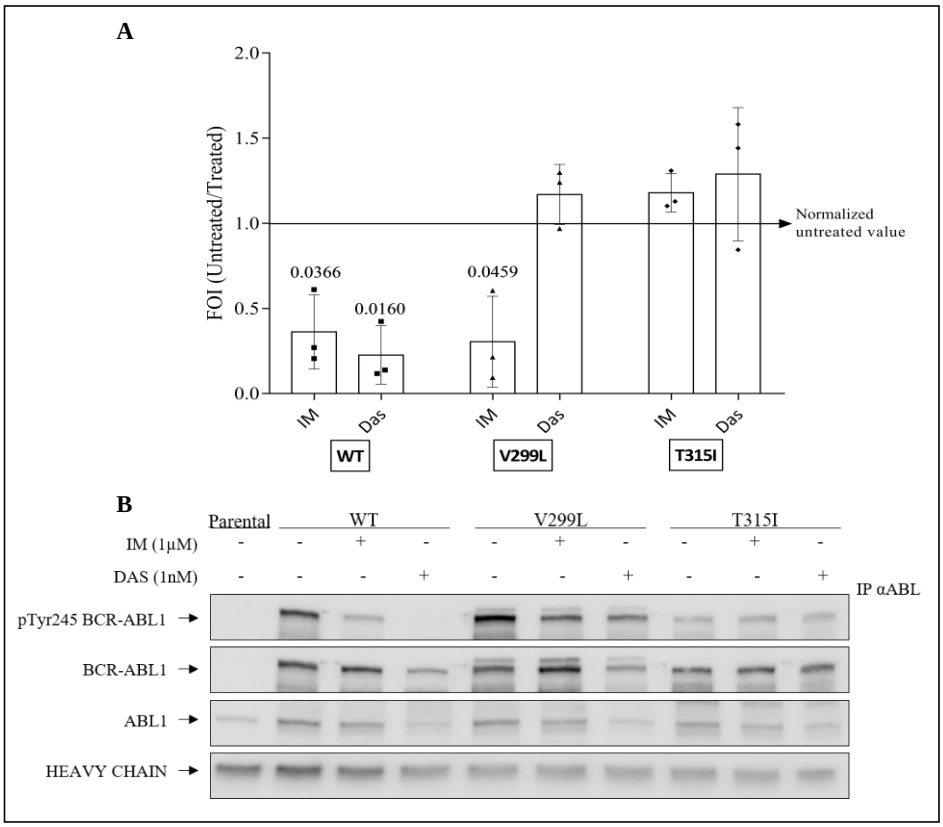


Figure 9 Kinase activity in BCR-ABL1 mutants.
A) Transfected mouse-derived pro-B Ba/F3 cell lines were treated in-vivo with 1μM Imatinib (IM) and 1 nM Dasatinib (Das) for 12h, while an aliquot of each cell type together with the non-transfected Ba/F3 were maintained without TKIs as control. Plot represent three independent experiments in which the kinase activity measurements were normalized to total BCR-ABL1 protein amount detected by Western blot on immuno-precipitated samples. Paired t-test was applied with a significant p value (<0.05). **B)** Examples of Western blot analysis of immunoprecipitated BCL-ABL1 and variants from experiments shown in panel A.

Here, we have verified the capability of the test to evaluate the TKIs action in BCR-ABL1 mutants featuring different degrees of resistance to TKIs. Mouse-derived pro-B Ba/F3 transfected cells stably expressing the human wild type p210 BCR-ABL1 (all drugs-sensitive), the V299L BCR-ABL1 mutant (only Dasatinib resistant) and T315I BCR-ABL1 mutant (drug-resistant to both drugs) were used as a models. They were all cultured in presence of specific TKIs as indicated by Redaelli et al.[68] (**Table 2**). In pro-B Ba/F3 carrying the wild-type variant of BCR/ABL1, Y245 dephosphorylation of native BCR-ABL1 protein, is followed by a decrease in kinase activity, whereas in resistant mutants (V299L, Dasatinib resistant, and T315I resistant to both TKIs) the reduction in activity was observed only for Dasatinib in the V299L mutant as expected (**Figure 9A and B**). No activity was recorded in the Ba/F3 parental cells (control) carrying only ABL1 protein. The BCR-ABL1 activity measured with our kinase assay thus reflects the drug action allowing the identification of drug-responsive and drug-resistant cells.

		IC_{50} fold increase (WT = 1)			
		Bosutinib	Imatinib	Dasatinib	Nilotinib
	Parental	38.31	10.78	> 50	38.43
	WT	1	1	1	1
ATP binding region	V299L	26.10	1.54	8.65	1.34
	T315I	45.42	17.50	75.03	39.41

Sensitive	≤ 2
Moderately resistant	2.01-4
Resistant	4.01-10
Highly resistant	> 10

TABLE 1 Indicates the IC 50 values of the TKIs (Imatinib and Dasatinib) used in the study.

An innovative procedure to preserve BCR-ABL1 protein from degradation

Historically, only protein denaturing approaches have been described that are suitable to inhibit the protein degradation occurring in primary myeloid cells, all of which, prevent the analysis of important biochemical parameters of the targets[64,65]. Nowadays, denaturing-boiling buffers are still used to study BCR-ABL1 expression in primary cell samples[69].

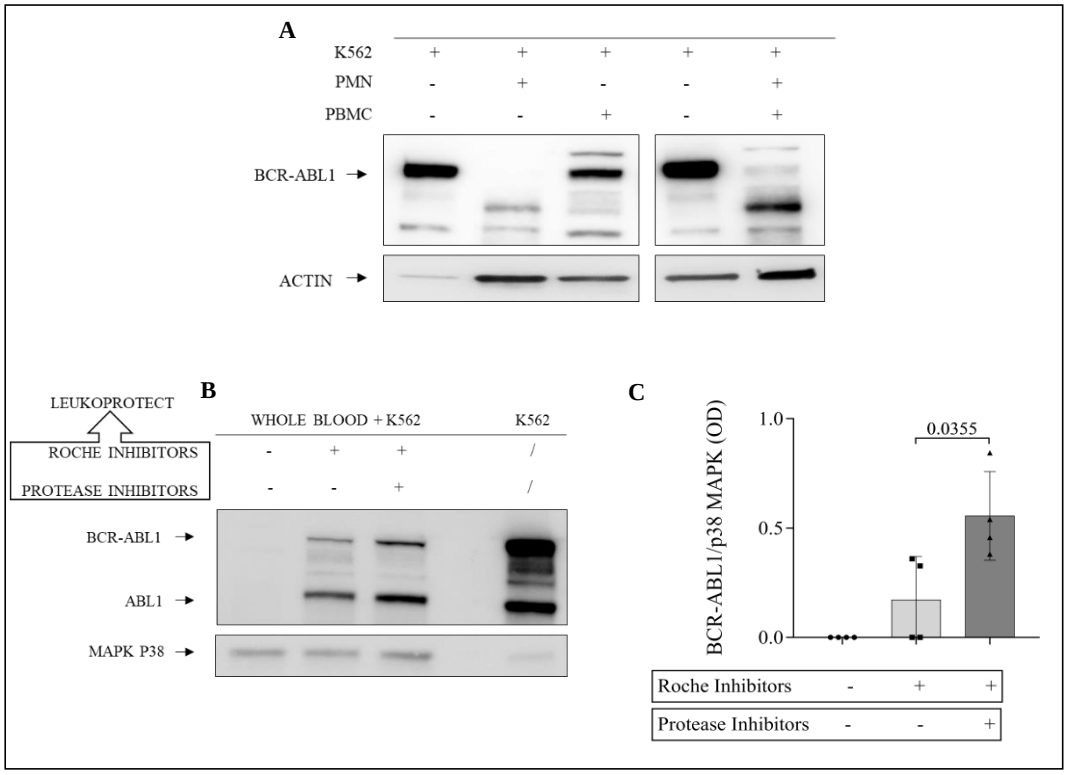


Figure 10 BCR-ABL1 degradative activity present in primary leukocytes and effect of protease inhibitor combinations (Leukoprotect)

A) PMNs and PBMCs cells were collected from healthy donor blood. Each cell population was spiked with BCR-ABL1-positive cell line (K562) in ratio 10:1. The cell mixes were lysed using a normal non-denaturing lysis buffer (as indicated in Materials and Methods) and post protein quantification with the Bradford technique, western blot analysis was performed. PMN fraction contain the degradative activity B) Healthy whole blood sample combined with K562 (ratio 10: 1) were pretreated with Roche cOmplete™ ULTRA Tablets inhibitors to whom a mix of further protease inhibitors were added (+) to increase BCR-ABL1 protein preservation. K562 cell lysate was used as reference. C) Quantification of the protective effect of protease inhibitors combination (Leukoprotect) on BCR-ABL1 protein maintenance shown in panel B. Unpaired t-test was applied with a significant p value (<0.05).

PMN degranulation is one of the mechanisms leading to release of multiple enzymes belonging to several protease family (serine-proteases, matrix metalloproteinase-MMP, and many others) once released, they act on many protein substrates including kinases. In particular on BCR-ABL1 that appear especially sensitive to their action[62] (**Figure 10A**).

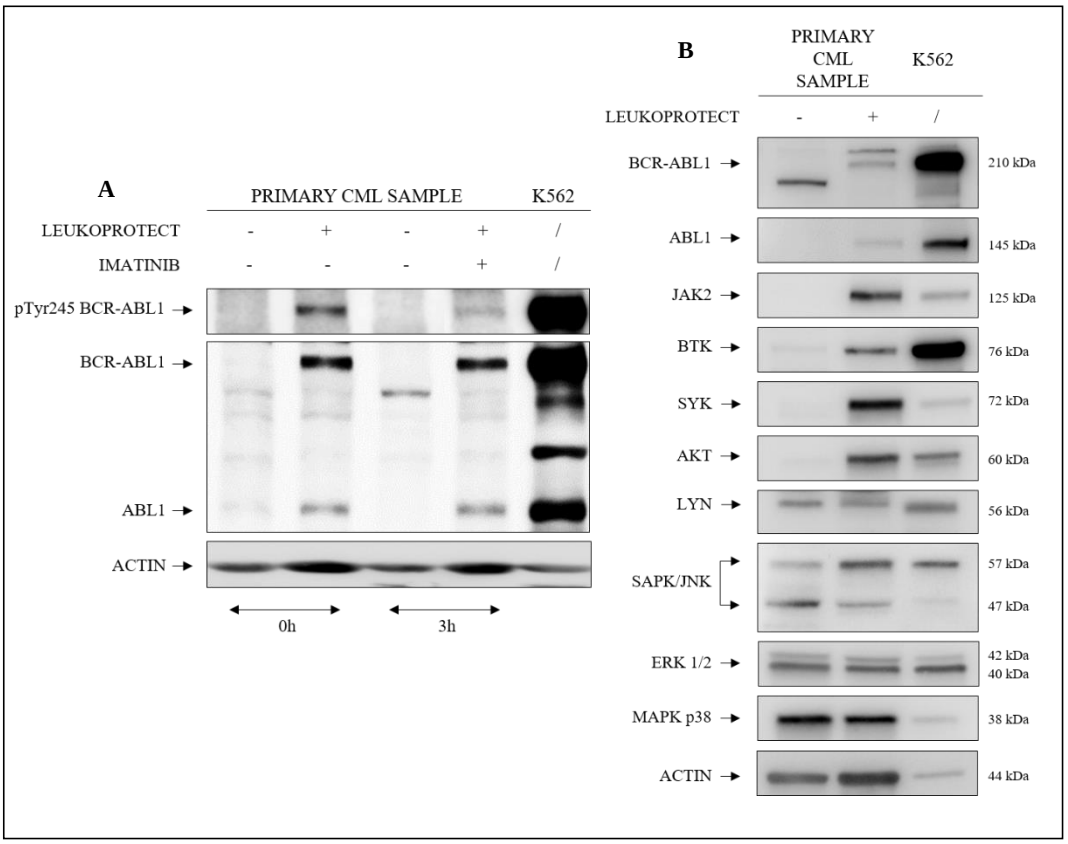


Figure 11 Leukoprotect composition blocks degradative action on BCR-ABL1 and other kinases of interest in CP-CML-derived primary cell samples.
A) Leukoprotect buffer pretreatment preserve BCR-ABL1 from degradation in CP-CML blood leukocytes. Representative experiment from a series of ten blood samples pretreated to maintain the protein of interest. An anti-pY245Abl and anti-abl1 total Western blot shows one of primary samples treated (+) using the Leukoprotect buffer or (-) untreated retrieved from patients before treatment and after 3h from the assumption of 400 mg Imatinib. Last lane is K562 cell lysate loaded as reference. B) Leukoprotect maintain several kinases from degradation. Western blot analysis of cleared lysates from CP-CML pre-treated (+) or not (-) with Leukoprotect before lysis. Membrane was probed with the specific antibodies indicated in **Table 1**. K562 cell lysate is loaded as reference.

Subsequently, we have identified a limited number of proteases that might be specifically involved in BCR-ABL1 protein degradation and within a collaborative project with AB Analitica (Padova, Italy) we devised a suitable protocol to protect BCR-ABL1 and other sensitive substrates from PMN proteolysis. Particularly, protease inhibitors use alone in non-denaturing cell lysis phase was not sufficient to prevent the protease activity on BCR-ABL1 (**Figure 10A**).

Thereby, we introduced a pre-lysis treatment phase with protease inhibitors, in order to preventively block the action of these released degradative enzymes. Testing Roche Inhibitors (cOmplete™ ULTRA Tablets) added to carefully selected protease inhibitors, we developed a pre-treatment buffer (Leukoprotect buffer) preventing the degradation of our protein of interest. Leukoprotect was established first in a simulated CML model that consist in a mixing of healthy donor blood with BCR-ABL1-positive K562 cell line in a 10:1 ratio (**Figure 10B**).

Thereafter, we moved on with primary CP-CML cells, applying the same protocol to different patients. Likewise, Leukoprotect buffer enabled us to recover the native BCR-ABL1 protein and to preserve its activity regulatory sites such as tyrosine-Y245 from primary cells (as showed in **Figure 11A**). In addition, Western Blot analysis of CML primary cells demonstrated a degradation on several kinases like JAK2, BTK, SYK, AKT which was completely preserved using our procedure, while other kinases (MAPK p38, ERK1/2, LYN) did not require the specific treatment procedure as they result resistant to degradation in presence of a standard mixture of protease inhibitors (**Figure 11B**). This observation underlines the importance to ensure a full protection of sensitive substrates to degradative activities that might be masked by a partial inhibition in presence of standard mixture of protease inhibitors. Together, these data not only show for the first time the possibility to preserve and analyse BCR-ABL1 (and many other protease-sensitive substrates) in native conditions also in blood leukocytes but emphasize the importance of protocol to preserve BCR-ABL1 as well as other important substrates (including other protein kinases) in primary CP-CML cell samples lysed in non-denaturing conditions.

BCR-ABL1 enzymatic activity in CML-model and in primary Philadelphia-positive leukemia samples

After the successful recovery of intact fusion protein, we set to confirm the functional integrity by evaluating its kinase activity.

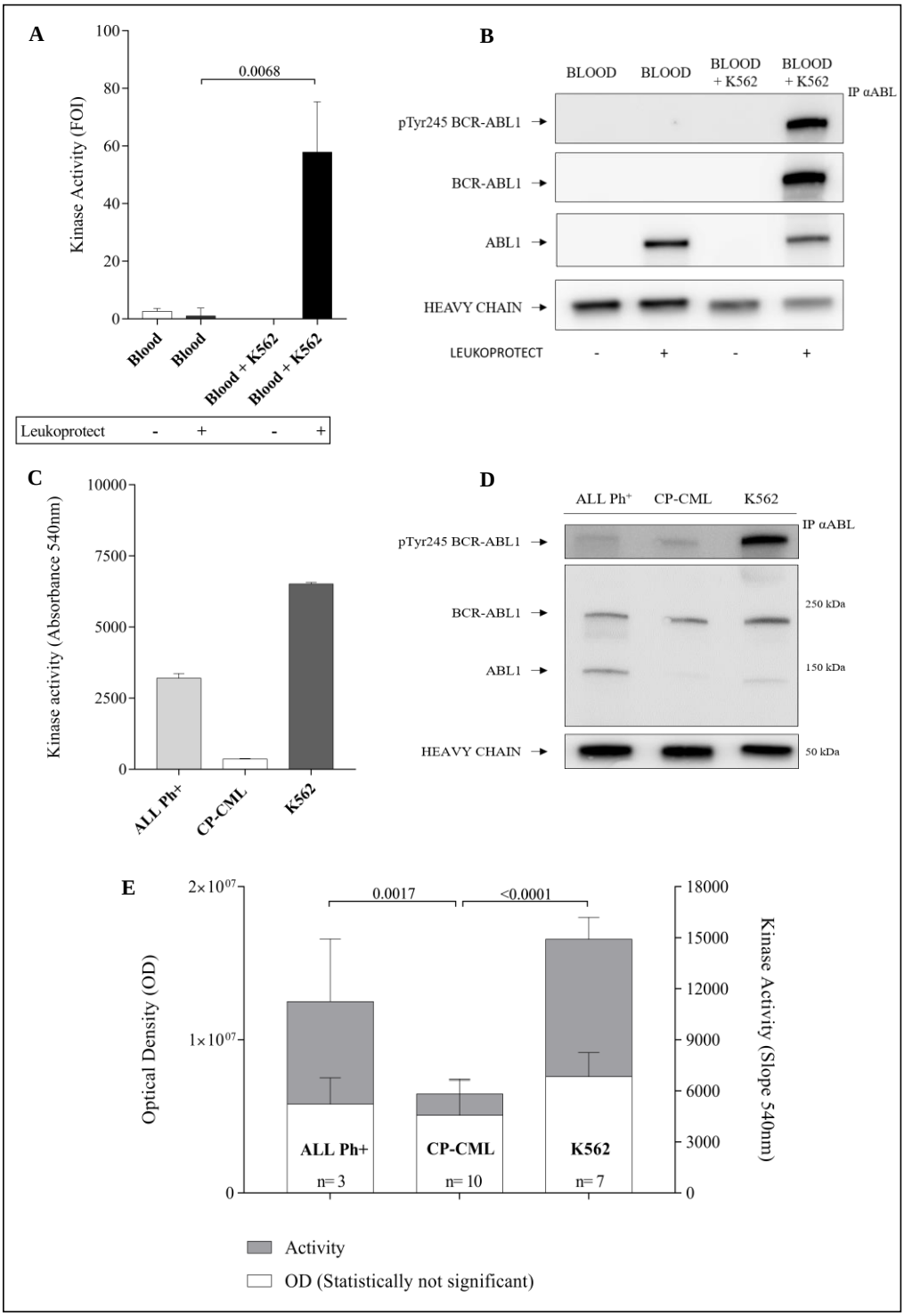


Figure 12 Enzymatic function preserved in BCR-ABL1 pretreated protein and investigation of BCR-ABL1 activity from chronic phase CML and ALL Ph+ blood samples. A and B) Immuno-precipitates obtained from healthy donor blood and the same blood samples mixed with K562 to simulate CML ware processed with (+) or without (-) pretreatment protocol and tested on for specific kinase activity using the Abl substrate peptide. Kinase activity graph (left) and Western blot analysis (right) underline preservation of both BCR-ABL1 protein integrity and function only after pre-treatment procedure. Paired t-test was applied before normalization with a significant p value (<0.05). **C and D)** BCR-ABL1 kinase activity from 13 patient samples (including 10 CP-CML and 3 ALL Ph+) along with K562 (7 replication). An example of the three different samples is shown in Figure 11B. **E)** The values obtained were then normalized on immuno-precipitated protein amount for the investigation. Densitometry analysis was carried out with the ImageJ software on western blot membrane probed with ABL1-antibody. Unpaired t-test was applied with a significant p value (<0.05).

For this purpose, we performed kinase assay on immuno-precipitated BCR-ABL1 starting from cellular lysates derived from a mix of healthy donor blood + K562 (ratio 10:1) obtained using Leukoprotect protocol. In the CML simulation condition, the oncoprotein activity was recorded only when BCR-ABL1 protein was preserved, confirming that we successfully isolated an intact and functional enzyme (**Figure 12A and B**). On the other hand, in healthy donor blood alone, the ABL1 protein was present but its kinase activity was undetectable, confirming that its activation state was tightly regulated.

After setting the protocol, we moved on measuring BCR-ABL1 expression and activity in primary cells from patients affected by CP-CML. We performed immunoprecipitation using 500 µg of total protein obtained from cell lysates starting from the evidence that in Chronic Phase the fusion protein was not over-expressed as is in the terminal blast-phase[16]. CML patients show a barely detectable kinase activity of BCR-ABL1 when compared to the same protein activity obtained when analysing the K562 cell line (**Figure 12C and D**). We therefore wanted to verify that this was not a general feature of BCR-ABL1 isolated from primary cells and thus performed the same analysis on BCR-ABL1 immuno-precipitated from Philadelphia-positive Acute Lymphoblastic Leukemia samples (Ph+ ALL) either in p210 and p190 protein variants. The kinase activity in these samples was readily

detected with specific value that was similar to the one measured in the blast crisis-derived K562 cell line (**Figure 12E**). These results confirm the suitability of the protocol to preserve BCR-ABL1 integrity and functionality in primary leukocytes and underline a previously unappreciated down-regulation of enzymatic activity in peripheral blood cells isolated from chronic phase CML.

VII. DISCUSSION

Once CML was associated to a deregulate activity of the protein tyrosine kinase BCR-ABL1, this was immediately considered an excellent target to reach in patients, by using tyrosine kinase inhibitors (TKIs). The efforts put in this vision lead to the development of drugs, whose progenitor is Imatinib, resulting in an outstanding increase in the quality and duration of life in CML patients that today is approaching those of age-matched controls. Nevertheless numerous efforts to produce increasingly powerful analogous molecules, the risk of resistance and disease relapse account key concepts on which it is worthwhile investing further efforts to finally eradicate this disease. Numerous evidence have shown that CML patients under therapy must strictly continue the TKI assumption to avoid relapse, underlining the existence of an intact leukemic potential requiring a continuous suppression of BCR-ABL1 activity. This proposal, already hypothesized in the past but very clear nowadays, is explained by a reported independence from BCR-ABL1 kinase activity in Leukemia stem cells[37,71]. In addition the indolent nature of the chronic phase of the disease where the leukocyte maintain a relatively normal morphology and function at variance with the blast transformation and the very aggressive behaviour of the blast crisis (BC) point to the need of additional events that modify the evolution of the disease. We have previously shown how at least a two-step model of evolution of this disease better fit the epidemiological data[72] in comparison with a single-step model[73].

A different level of BCR-ABL1 kinase activity, being phase-disease related has never been measured directly due to the technical limitations previously described. In order to overcome these limitations, we combined recent advances in peptide biosensor-based assay, allowing an accurate measure of enzymatic activity with a modified procedure of leukocyte proteases inhibition in order to preserve native BCR-ABL1 protein during cell lysis performed under non-denaturing conditions. BCR-ABL1 activity was detected by reacting the isolated kinase on a highly specific substrate for c-Abl which contain Src-homology 3 (SH3)-binding ligand to enhance interaction with the kinase itself [67]. Placzek et al. demonstrated substrate

specificity on the BCR-ABL1-phosphorylative detection capability in a cellular model overexpressing a dysregulated variant of ABL1[74]. By exploiting an immortalized BCR-ABL1-positive cell model, we showed a capability of the test to detect kinase function that was not present in negative BCR-ABL1 models. The test robustness was verified by analysing some properties such as: data repeatability and procedure sensitivity and specificity. During the '90s many studies ascertained a degradative action on proteins belonging to blood primary cells especially in mature myeloid cells population, which was absent in the immature counterpart (CD34+) or in leukemia blast cells[62]. It is quite clear that a protease component would operate in this process, being released during myeloid cells lysis and compromising the integrity of studied proteins[63]. Despite these observations, no progress in the field has been reported so far. Taking advantage of our background in protein and kinase/phosphatase studies we set to curb this strong degrading action through whole blood treatment with a precisely formulated mixture of protease inhibitors dissolved in a physiological buffer (PBS).

It is important to underline how these proteases take action on multiple substrates, as seen in Figure 10B, where the consequences of cell lysis on various other kinases present in CML blood sample is shown. Essentially, they disappeared if samples are not pre-treated with any protease inhibitor buffer, suggesting the presence of a specific enzymatic cleavage sites in these substrates that become accessible to proteases once they are released from the cell compartment, to which they are restricted before lysis. Notably this degradation occurs almost immediately after lysis.

This project lead to the development of a protocol suitable to record for the first time the kinase activity in Chronic-Phase CML patients samples using a peptide biosensor-based assay. The possibility to identify BCR-ABL1 enzymatic activity directly from patients opens up a greater understanding of the biology in this early stage of the disease. Particularly, the CP-CML primary samples, when normalized against the amount of BCR-ABL1 tested and compared with both a blastic-phase cell model and primary samples of Ph+ ALL (a more severe form of leukemia), showed strongly reduced values of phosphorylation of the reporter peptide. To elucidate these results, it will be necessary to further investigate the state of this

kinase. It is well documented that presence of mutations in protein structure can produce greater kinase activity. This could explain the different results with the blast crisis model. However, this is not the case for K562 and LAMA84 cell lines, which harbour an unmuted BCR-ABL1 gene sequence. Likewise, it is possible to speculate on a transitory state of reduced kinase activity that is detectable also in the isolated enzyme that, however, has never been described so far.

In summary, this original protocol allowed to measure BCR-ABL1 kinase activity in newly diagnosed CML samples demonstrating a very low activity state of the kinase in this early disease phase. This observation also suggest that studies performed with transfected cell lines do not precisely recapitulate the signalling events occurring in primary cells in the chronic phase of the disease, pointing to the need to reinvestigate the role of BCR-ABL1 in the context of primary cells. Indeed, the role of additional, kinase independent events is well described in the literature and has been associated with the presence of additional genetic lesions[75]. Furthermore, a kinase-independent role of BCR-ABL1 in leukemia stem cells would imply that drugs that target BCR-ABL1's scaffolding ability or its DNA-binding ability should be used in conjunction with current therapeutic regimens to increase their efficacy and eradicate the stem cells of chronic myeloid leukemia[76]. In this regard, our observations that a tyrosine phosphatase is specifically downregulated in CML, and that its re-expression is induced in the same patients when the neoplastic population is wiped out by the treatment suggest that physiological regulations of BCR-ABL1 activity are active in at least certain stages of the disease[77,78]. The set-up of a reproducible kinase assay in a relatively simple ELISA format can permit the detection and the follow-up of this kinase's activity in its native state and can contribute to shed light regarding this important issue. Notably, the assay format can be extended to other kinases for which substrates and suitable antibodies for immunoprecipitation are available. This would open up the possibility to rapidly and precisely appreciate in vitro and in vivo the effects of TKIs used in clinic with applications in primarily, but not limited to, haematological malignancies.

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