Original Article
Effects of Ibrutinib on biophysical parameters of platelet in patients with chronic lymphocytic leukaemia

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Abstract: Patients with chronic lymphocytic leukemia (CLL) treated with Ibrutinib often present hemorrhagic complications. Platelets dysfunction is well documented by aggregometry and flow cytometry, but the mechanisms by which Ibrutinib treatment influences the platelets status is yet to be evaluated. The aim of this study is to identify platelet membrane parameters in chronic lymphocytic leukemia (CLL) that could be altered by Ibrutinib administration. In this paper we propose a set of fluorescence measurements of the following parameters: membrane fluidity, resting membrane potential, and reactive oxygen species production of platelets suspensions obtained from CLL patients treated or not with Ibrutinib as markers for platelets status in this pathological situation. Platelets from CLL patients treated with Ibrutinib have higher membrane fluidity, lower resting membrane potential and higher level of reactive oxygen species production compared to the untreated CLL patients. These patients are also presenting higher membrane fluidity and lower resting membrane potential compared to healthy volunteers.

Keywords: Ibrutinib, leukemia, platelets, membrane potential, ROS

Introduction

Patients with chronic lymphocytic leukemia (CLL) have usually associated a high risk of hemorrhagic complications which may be induced by anticoagulation or antiplatelet therapy (possibly associated with renal impairment, anemia, thrombocytopenia, or alcohol abuse) [1].

An increased activity of Bruton’s tyrosine kinase (BTK) has been found increased in B-cell malignancies such as CLL [2]. The BTK has a signaling role in platelet (PLT) activation via phospholipase Cy2, by reducing the expression of the PLT membrane glycoprotein VI (GPVI). The PLT adhesion to collagen is realized through GPVI, and, once the adhesion occurred, a signaling cascade (involving Src kinases Fyn and Lyn, tyrosine kinase Syk, and Tec kinases BTK) is triggered, leading to the PLT activation [3, 4].

Ibrutinib, a BTK inhibitor used in CLL treatment, was associated with unexpected serious bleedings [2, 5]. Platelet dysfunction, as diagnosed by platelet aggregation tests, was identified as the main cause of bleeding in CLL patients treated with Ibrutinib [6-8]. Indeed, irreversible inhibition of BTK by Ibrutinib was associated with a decrease of PLT activation, resulting in a reduction of their aggregation capacity, as found in studies on PLTs from healthy persons or nonhuman primates [9].

The properties of platelet membrane modulate the activity of intrinsic proteins (like channels, pumps, signaling proteins, etc.) and expression of receptors [10, 11].

Even though the pro-hemorrhagic effect of Ibrutinib is well documented [12], the way by which Ibrutinib influences the PLT membrane status is yet to be evaluated.
In this study we propose a set of biophysical parameters which can be relevant for the PLTs impairment in CLL patients treated with Ibrutinib. Fluorescence measurements of the following parameters: membrane fluidity (MF), resting membrane potential (RMP), and production of reactive oxygen species (ROS), were done on PLTs suspensions obtained from CLL patients treated or not with Ibrutinib. Our data may complement the information given by the standard techniques used to monitor the PLT activity (aggregometry and flow cytometry).

Materials and methods

Selection of patients

The study included 36 patients with CLL from the Hematology Department of Colentina Clinical Hospital and 16 healthy volunteers as controls.

Inclusion criteria were:

1) Male or female > 18 years of age.

2) Diagnosis of B-cell CLL, established according to International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria.

3) CLL patients treated with single-agent Ibrutinib must be first-line (patients ≥ 65 years old or with presence of 17p/TP53 mutation at FISH analyses) or relapsed/refractory (one or more prior therapies) CLL/SLL treatment settings. Patients must have accepted IWCLL criteria for initiation of therapy.

4) Patients in untreated group must be included in watch and wait group (never treated before enrolment).

5) Karnofsky performance score of ≥ 60.

6) Required baseline laboratory data (within 4 weeks prior to enrolment).

Required screening laboratory values:

- Platelets > 100x10^3/mL
- Hemoglobin > 10.0 g/dL
- Renal creatinine clearance > 40 ml/min
- Pregnancy-HCGc negative for women in fertile period
- CRP (reactive protein C) < 1.5 N
- Normal serum glucose, cholesterol, triglycerides and lipids

7) Signed informed consent indicates the acceptance of the subject regarding aspects of participation to the study.

Exclusion criteria were:

1) Known histological transformation from CLL to an aggressive lymphoma (Richter transformation).

2) History of a non-CLL active malignancy (solid tumor or myeloproliferative neoplasms, myelodysplastic syndromes) with ongoing treatment or less than 6 months after chemotherapy.

3) Prior or ongoing clinically significant illness, medical condition, surgical history, physical finding, or laboratory abnormality that could adversely affect the assessment of study results (dyslipidemia, diabetes mellitus, arterial hypertension stroke or myocardial infarction or arterial/vein thrombosis or pulmonary embolism).

4) Evidence of ongoing systemic bacterial, fungal, or viral infection at the time of enrolment in the study.

5) Ongoing alcohol or drug addiction.

All participants have given informed consent. The procedures used in the study were approved by the Hospital Ethics Committee. The diagnosis of CLL was established using the criteria proposed by the consensus guidelines of the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) [13]. CLL patients were divided into two subgroups based on the treatment received: treated with Ibrutinib (26 patients) and untreated (10 patients). The patients treated with Ibrutinib were receiving the drug for a maximum period of 18 months 420 mg daily, while untreated patients were on “watch and wait” status, never receiving any CLL medication. The patients were not presenting any hemorrhagic complications prior enrolment in the study. The control group had the median age of 51 years (min 29, max 74) and included 37.50% males.

The clinical and hematological characteristics of the patients are presented in Table 1.
Preparation of PLTs suspensions

The protocol for the preparation of PLTs suspensions was designed to obtain non-activated platelets. The PLTs are very sensitive: they activate and aggregate easily under mechanical forces (e.g., fast pipetting, vigorous shaking or long time centrifugation), in presence of calcium and on contact with glass; therefore all these conditions have been avoided during the PLTs preparation. Blood sampling was performed in the morning (after overnight fasting). Blood was collected in sodium citrate tubes by venipuncture. Platelet rich plasma (PRP) was obtained by gentle centrifugation at 130 × g for 10 minutes, at room temperature. PLTs were separated from PRP by gel-filtration chromatography on a Sepharose CL-2B column (Sigma, 2B300) in Tyrode buffer without calcium, with glucose (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.34 mM Na₂HPO₄, 1 mM MgCl₂, 10 mM HEPES, 5 mM D-glucose) [14]. This chromatographic separation of PLTs has the advantage to avoid mechanical activation of platelets which may occur when PLTs are pelleted by standard centrifugation. Moreover, 0.2 U/mL Apyrase (Apyrase grade I from potato, Sigma, A6132-5KU) were added to prevent PLTs aggregation during the manipulation. The optical density (OD) of PLTs suspension measured at 355 nm was adjusted to 0.8 ± 0.04 with Tyrode buffer. PLTs suspension was then split in equivalent samples for the following measurements: resting membrane potential (RMP), membrane fluidity and production of reactive oxygen species (ROS).

Membrane fluidity measurements

PLTs membrane fluidity was assessed by fluorescence anisotropy using N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH) (Sigma, 43060) (10 mM stock solution in dimethyl formamide kept at -20°C). TMA-DPH is a rigid molecule with an electronic transition moment very well defined against the geometry of the molecule. The fluorophore molecules insert rapidly into the lipid bilayer (1 min) and remain trapped within the membrane for about 25 min [15]. When the sample is illuminated with polarized light, only those molecules that have the transition vector aligned parallel to the polarized excitation electric vector have the highest probability of excitation. The emitted light will be less polarized, depending on the movement possibilities of the fluorophore within the membrane during the fluorophore excited state lifetime. The degree of depolarization is quantified by a parameter called fluorescence anisotropy (r), defined as:

\[ r = \frac{l_{hv} \cdot Gl_{hv}}{l_{hv} + 2Gl_{hv}} \]
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where G represents a correction factor \((G = \frac{I_{HV}}{I_{HH}})\) and \(I\) stands for the fluorescence intensities with excitation and emission polarizers oriented as specified by the indices (V for vertical, H for horizontal). The more fluid the lipid bilayer, the lower the anisotropy parameter \(r\) values.

TMA-DPH was added to the PLTs suspension (final concentration of 50 μM), incubated for 2 min at 37°C and fluorescence anisotropy \((\lambda_{ex} = 355 \text{ nm}, \lambda_{em} = 425 \text{ nm})\) was measured.

Resting membrane potential measurements

RMP was assessed by fluorescence, using the probe 3,3 Dipropylthiadicarbocyanine 5 (DiSC3-5) (Invitrogen, D306), 0.5 mM stock solution in dimethyl formamide, kept at -20°C [16]. The cationic dye DiSC3-5 is membrane permeant and distributes between cytoplasm and extracellular medium according to the electrochemical gradient: the more negative the interior, the higher the cytoplasmic concentration of the fluorophore. A specific feature of DiSC3-5 is that its fluorescence quantum yield is much smaller inside the cell than outside. Thus, as the probe accumulates in the cell, the fluorescence intensity drops, reaching a plateau at a level characterizing the membrane potential. To transform the fluorescence intensity in units of mV, the membrane potential is clamped to the potassium electrochemical gradient by adding the specific potassium transporter Valinomycin. While \(K^+\) is leaking out of the cell, the membrane hyperpolarizes, more DiSC3-5 enters the cell and its fluorescence decreases to a new plateau. Then, as known concentrations of \(K^+\) are added outside the cell, the fluorescence intensities are reaching new plateau levels. The membrane potential is computed (for each plateau) using the Nernst equation:

\[
E = \frac{RT}{ZF} \ln \left[ \frac{[K^+]_{out}}{[K^+]_{in}} \right]
\]

where \(E = \text{PLT resting membrane potential (mV)}, \ R = \text{universal gas constant} (8.314 \text{ J.mol}^{-1}.\text{K}^{-1}), \ T = \text{absolute temperature (K)}, \ z = \text{potassium ion relative electric charge (+1)}, \ F = \text{Faraday constant (96485 C.mol}^{-1}), \ [K^+]_{out} = \text{added (known) potassium concentration outside the cell}, \ [K^+]_{in} = \text{basal cytoplasmic concentration of potassium (135 mM)}\). The computed membrane potential is represented as a function of the fluorescence intensities and fitted with a linear equation that allows the interpolation of fluorescence intensity corresponding to the first plateau.

DiSC3-5 at final concentration 2 μM was added to the PLTs suspension incubated at 37°C until reaching the fluorescence signal plateau \((\lambda_{ex} = 620 \text{ nm}, \lambda_{em} = 670 \text{ nm})\). Valinomycin (Sigma, V0627) (50 mM stock solution in ethanol kept at -20°C) was then added to the PLTs suspension (final concentration of 50 μM) and a new fluorescence plateau was obtained. Aliquots of 2 M KCl (Sigma) were added sequentially after each new plateau was obtained.

ROS production measurements

ROS levels were assessed by fluorescence using 2’,7’-Dichlorofluorescein diacetate (H2DCFDA) (Sigma Aldrich, 35845) (0.5 mM stock solution in methanol kept at -20°C). H2DCFDA is a membrane permeant, nonfluorescent compound which is deacetylated by cellular esterases after entering the cell, oxidized by ROS to a highly fluorescent compound (DCF). The fluorescence intensity is thus proportional to the amount of ROS produced by the cell.

H2DCFDA was added to the PLTs suspension (final concentration of 5 μM and fluorescence intensity \((\lambda_{ex} = 488 \text{ nm}, \lambda_{em} = 523 \text{ nm})\) was measured continuously for 15 minutes (at 37°C). ROS production was assessed by normalizing the area under the fluorescence intensity curve to the OD of the sample.

All fluorescence measurements were done using a Horiba Jobin Yvon FluoroLog 3 spectrofluorometer (with two emission channels) s(USA).

Statistical analysis

Statistical analysis of data was performed using Mann-Whitney rank sum test (two variables) or Kruskal-Wallis test (more than two variables) for variables with abnormal distribution. The statistical significance was set for \(P = 0.05\). For all statistical computations, FluorEssence™ 2.1 (developed by Horiba under OriginPro™) was used.
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Results

Fluorescence anisotropy of PLTs membrane

The fluorescence anisotropy of PLTs membrane was found to be: median value for controls $r = 0.096$ (min 0.077, max 0.141), median value for untreated CLL patients $r = 0.084$ (min 0.069, max 0.141) while for patients treated with Ibrutinib, median $r = 0.073$ (min 0.040, max 0.121) (Figure 1). CLL patients treated with Ibrutinib have statistically higher fluidity of PLTs membrane (lower fluorescence anisotropy) as compared to CLL untreated patients ($P = 0.010$). There is a tendency (although not statistically significant) of an increased membrane fluidity in case of untreated CLL patients as compared to controls.

Resting membrane potential of PLTs membrane

An example of an experimental curve and the corresponding RMP calibration using the Nernst equation for potassium clamped potential are presented in Figure 2.

The median value of the RMP for controls was found to be -56 mV (min -37 mV, max -68 mV). The untreated CLL group presented a RMP median value of -58 mV (min -39 mV, max -72 mV), without statistical difference as compared to controls. The RMP for Ibrutinib treated CLL patients was -43 mV (min -21 mV, max -58 mV), with a statistically significant difference when compared to any of the previous two groups ($P < 0.05$) (Figure 3).

Evaluation of ROS production by PLTs

An example of an experimental curve of ROS production is presented in Figure 4.

Higher levels of ROS production were observed for PLTs from patients treated with Ibrutinib: median value $6.12 \times 10^{10}$ (min $4.17 \times 10^{10}$, max $2.07 \times 10^{11}$) compared to untreated patients: median value $4.65 \times 10^{10}$ (min $1.08 \times 10^7$, max $5.85 \times 10^{10}$), with a statistically significant difference between these groups ($P = 0.014$). In case of untreated CLL patients, there is a slightly lower ROS production as compared to controls (median value $5.52 \times 10^{10}$, min $3.94 \times 10^{10}$, max $1.58 \times 10^{11}$) (Figure 5).

Discussion

Membrane fluidity as seen by fluorescence anisotropy is a membrane parameter which quantifies the wobbling movements of the lipid tails within the membrane bilayer. Alteration of these movements was shown to be correlated to the expression and activity of various PLTs membrane receptors [16-19].

There are few reports regarding the complex correlation of the membrane fluidity and the receptor expression on lymphocytes in CLL patients, having an impact on the defective transmembrane signaling [20-22]. To our knowledge, no reports are published regarding the relationship between platelet membrane fluidity and PLTs functions in CLL.

Kozubski et al. reported that Alzheimer patients present platelets with significantly increased membrane fluidity, when measured by Electron Spin Resonance technique. In the same time the PLTs reactivity was significantly decreased, since a reduced expression of GPIb in the resting and activated platelets was observed [17].

Vinik et al. found a decreased membrane fluidity in PLTs of diabetic patients which is associated with enhanced GP receptor binding of agonists [23].

In our study, we found that Ibrutinib treatment determined a statistically significant increase in the membrane fluidity of PLTs compared to...
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Figure 2. Measurement of resting membrane potential (RMP) in millivolts (mV). The experimental curve of the 3,3 Dipropylthiadicarbocyanine 5 (DiSC3-5) fluorescence intensity is shown in counts per second (CPS) (A). The RMP computed on the base of Nernst equation is represented as a function of fluorescence intensity (B).

Figure 3. Resting membrane potential (RMP) in millivolts (mV) measured on platelets of controls and chronic lymphocytic leukemia patients untreated and treated with Ibrutinib (Statistical significance * = P < 0.05).

Figure 4. Measurement of reactive oxygen species production. Experimental curve of the Dichlorofluorescein fluorescence intensity (in counts per second - CPS) is presented and the area under the curve is computed.

Figure 5. Reactive oxygen species (ROS) production measured on platelets of controls and chronic lymphocytic leukemia patients untreated and treated with Ibrutinib. The ROS production is proportional to the area under the curve (AUC) measured in counts of fluorescence per optical density (OD) of the sample, as shown in Figure 4 (Statistical significance * = P < 0.05).

untreated patients (Figure 3). Compared to controls, the untreated patients presented a slight increase of membrane fluidity, although not statistically significant.

Even though we cannot propose a molecular mechanism, we may speculate that the membrane fluidity is modulating the expression of PLTs membrane receptors.

Moreover, it is known that Ibrutinib is modifying the expression of receptors GPVI and GPIbα [2, 12]. In case of our patients treated with Ibrutinib, the increased membrane fluidity may down regulate the expression of receptors, as reported by above mentioned articles.
According to Friedhoff and Sonenberg [16], the membrane potential of normal human platelets was estimated to be -52 to -60 mV. In our study the median value of RMP of controls was -56 mV in good agreement with the data reported in literature. In case of CLL patients we obtained a median value of RMPs -58 mV (min -39 mV, max -72 mV) without statistical difference compared to controls. The administration of Ibrutinib to these CLL patients was found to be associated to a depolarization of the PLTs membrane: RMP median value of -43 mV (min -21 mV, max -58 mV) with a statistically significant difference when compared to controls and untreated patients (P < 0.05).

According to Smith et al., there are two types of potassium channels in the PLTs membranes: calcium-gated and voltage-gated channels. In the early stages of platelet activation, the PLTs potential is reported to hyperpolarize to approximately -80 mV, but later, it may depolarize due to an opening of various channels [24].

We do not know if the Ibrutinib is the cause of the PLTs depolarization found by our study in the treated patients, but we may speculate that it interferes with the intracellular calcium flux. In vitro studies on Jurkat cells, have shown that the pre-treatment with Ibrutinib diminishes the intracellular calcium flux in response to T cell receptor stimulation [25]. In a study using platelets from patients under Ibrutinib treatment, Bye and collaborators reported that Ibrutinib inhibits the Ca^{2+} elevation and platelets aggregation evoked by collagen-related peptide (CRP)-XL and collagen [4]. Moreover, it is also known that under Ibrutinib, the patients are presenting non active PLTs [12]. This status may be attributed to the shedding process induced by Ibrutinib on various membrane receptors. For instance, Ibrutinib produces a time- and dose-dependent shedding of GP1b-IX complex and GP1bblla, but not of GPVI and GPV [26]. Bye and collaborators [27] confirm the effect of Ibrutinib on the integrin, but they are also evidencing an inhibition effect of Ibrutinib on GPVI signaling, resulting in formation of unstable thrombi. They are attributing the inhibition effect of Ibrutinib to the clot retraction and signaling evoked by platelet adhesion to immobilized fibrinogen.

The production of ROS was found to be higher in CLL patients treated with Ibrutinib when compared to untreated ones (P = 0.014). All CLL patients treated with Ibrutinib included in this study were in refractory status (second or third line of therapy) or newly diagnosed (first line with associated high cytogenetic risk-TP53 or 17p mutations present). The difference in ROS productions between treated and untreated patients could be explained by these characteristics of the Ibrutinib group. Untreated patients were belonging to a low risk CLL group (no TP53 or 17p mutations or IGHV unmutated). Similar results were recently reported by D’Arena and collaborators [28]. Their CLL patients with advanced stages of disease or characterized by markers of disease aggressiveness were presenting an increased ROS production and decreased antioxidant defense mechanisms.

Oxidative stress was also considered as a prognostic factor of unfavorable disease evolution in some other reports in which a high ROS production was found in CLL patients [29]. Moreover, the mitochondrial metabolism and ROS production was shown to be elevated in CLL [30]. High level of ROS production is due to the mitochondrial respiratory chain alterations of cells in CLL: defective oxidative phosphorylation or an enhanced mitochondrial biogenesis. Another mechanism of increased ROS production is related to the modified activity of membrane-bound NADPH-oxidase (NOX) complex [31]. Presence of high levels of ROS induces genetic instability, development of drug resistance and less harness of cell signaling [31]. In case of pathological conditions like those of heart failure patients, Mondal and collaborators proved that high level of ROS production is associated with platelet apoptosis and depolarized platelets and induces bleeding complication [32]. Moreover, the integrin α2bβ3, GP1bα and GPVI shedding and platelet dysfunction have been associated with high ROS production by [33, 34].

In conclusion, platelets from CLL patients treated with Ibrutinib have higher membrane fluidity, lower resting membrane potential and higher level of ROS production compared to the untreated CLL patients. These patients are also presenting higher membrane fluidity and lower resting membrane potential compared to
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healthy volunteers. Ongoing experiments are intended to correlate these preliminary data with information about platelets activity as resulting from standard of care techniques in the attempt to find mechanistic explanation for our observations.

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Disclosure of conflict of interest

None.

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