Ibrutinib treatment affects collagen and von Willebrand Factor-dependent platelet functions

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Running Title: Ibrutinib affects platelet functions
Key points:
- Ibrutinib affects collagen and VWF-mediated platelet activation.
- The bleeding diathesis correlates with defects in collagen-induced platelet aggregation and firm adhesion on VWF at arterial shear rate.

Abstract
The oral Bruton’s tyrosine kinase inhibitor, ibrutinib, has recently demonstrated high efficiency in patients with relapsed B-cell malignancies. Occurrence of bleeding events has been reported in a subgroup of ibrutinib-treated patients. We demonstrate that ibrutinib selectively inhibits platelet signaling and functions downstream of the collagen receptor GPVI and strongly affects firm platelet adhesion on von Willebrand Factor under arterial flow. A longitudinal study of 14 patients indicated a correlation between occurrence of bleeding events and decreased platelet aggregation in response to collagen in platelet rich plasma and firm adhesion on von Willebrand Factor under arterial flow. Addition of 50% untreated platelets was sufficient to efficiently reverse the effects of ibrutinib, and platelet functions recovered following treatment interruption as physiological platelet renewal occurred. These data have important clinical implications and provide a basis for haemostasis management during ibrutinib treatment.
Introduction

The Bruton tyrosine kinase (Btk) is an essential actor downstream of the B-cell receptor (BCR) and its covalent inhibitor, ibrutinib, has recently been approved for therapies of relapsed chronic lymphocytic leukemia (CLL) and mantle-cell lymphoma (MCL).\textsuperscript{1-8} Bleeding has been reported in up to 50\% of ibrutinib-treated patients. Most events were of grade 1-2 (spontaneous bruising or petechiae) but, in 5\% of patients, they were of grade 3 or higher after trauma.\textsuperscript{4-6} Platelets are the most important blood cells to prevent bleeding after vascular injury. Two Tec family kinases, Btk and Tec, are involved in platelet activation downstream of the collagen receptor GPVI via phospholipase C\(\gamma\)\textsubscript{2} (PLC\(\gamma\)\textsubscript{2}) phosphorylation and activation.\textsuperscript{9,10} Under arterial shear rate, interaction of platelets with the damaged vessel wall is largely mediated by binding of von Willebrand factor (VWF) to its receptor, the GPIb-IX-V complex. In a mouse model, Btk has been shown to play a role in VWF/GPIb-IX-V-induced platelet activation.\textsuperscript{11} To further characterize the bleeding events in ibrutinib-treated patient we have investigated the effect of this drug on platelet functions in vitro, and ex vivo in fourteen patients.

MATERIALS AND METHODS

For in vitro experiments, whole blood, platelet rich plasma (PRP) or washed platelets from healthy donors free of anti-platelet medication were preincubated with ibrutinib (PCI-32765 from Selleckchem) or DMSO for 10 or 30 minutes as indicated. For ex vivo experiments, blood samples were obtained before and 2 to 4 weeks after starting ibrutinib treatment (Imbruvica\textsuperscript{®}, Janssen-Cilaq laboratories) in patients with CLL (420 mg daily) or MCL (560 mg daily)\textsuperscript{4-6} (French compassionate use program) after informed consent, in accordance with the Declaration of Helsinki. This study
was approved by the institutional review board of the Toulouse hospital. After exclusion of patients with antiplatelet therapy or a platelet count below 80 G/L, 14 patients were included, the characteristics of whom are reported in Table S1 in supplemental appendix. Experiments were performed in PRP prepared from citrated blood (aggregation) or heparinised whole blood (adhesion onto VWF). Most laboratory methods have been described previously and are reported in the method section of supplemental materials. Statistical analysis was performed using paired or unpaired Student’s test (Excel software, \(^* p<0.05\) and \(** p<0.01\)).

RESULTS AND DISCUSSION

Ibrutinib inhibits collagen and CRP-induced platelet aggregation and PLC\(_2\) phosphorylation in vitro.

At a clinically achievable dose (0.5 µM), \(^6,13\) ibrutinib had no impact on aggregation of washed platelets induced by the thromboxane A2 analogue U46619, thrombin related peptide (TRAP) or thrombin but dose-dependently inhibited collagen-induced platelet aggregation (Figures 1A-B and S1 in supplemental appendix). This effect was accompanied by the inhibition of PLC\(_2\) phosphorylation on the Btk-dependent phosphorylation site Tyr753.\(^{14}\) Low concentrations of ibrutinib, which strongly reduced PLC\(_2\) phosphorylation, had no effect on Src-family kinases activation (phosphorylation of Tyr416) and on the whole pattern of tyrosine phosphorylation in collagen-stimulated platelets. Higher ibrutinib concentrations (≥0.5 µM) consistently reduced Src-family kinases activation and the tyrosine phosphorylation of several proteins (Figure 1B-C). Selectivity tests of ibrutinib against a screening panel of kinases have shown that several Src-kinases may indeed be affected at this drug concentration in vitro.\(^3\)  Ibrutinib inhibited dose dependently Btk autophosphorylation
Ibrutinib inhibits platelet adhesion onto VWF under high shear rate in vitro.

Binding of VWF to GPIb-V-IX is required for tethering platelets to the injured vessel wall and for integrin-mediated platelet arrest under blood flow. VWF-GPIb interaction stimulates cytoskeletal reorganization in rolling platelets via a shear-sensitive signaling pathway linked to PLC and intracellular calcium mobilization.

Preincubation of blood from healthy donors with ibrutinib decreased the firm platelet adhesion onto VWF under high shear rate (Figure 1F) while sparing platelet rolling (Video 1 in supplemental appendix) and expression of GPIb (Figure S2A in supplemental appendix).

Haemostasis-related adverse effects of ibrutinib in CLL and MCL patients correlate with platelet dysfunction.

From 14 included patients, 5 displayed treatment-related haemostasis defects including spontaneous bruising, superficial bleedings or epistaxis (Figure 2A) while all had clinical response to the drug (Table S1 in supplemental appendix). Standard light transmission aggregometry in PRP indicated that ibrutinib treatment had no or very minor effect on ADP or U46619-induced platelet aggregation, in all patients tested. Seven patients presented a significant defect in platelet aggregation to collagen. All
patients with bleeding diathesis presented a strong inhibition of collagen-induced aggregation in PRP. The firm platelet adhesion onto VWF was analyzed in 6 patients. Platelets from patients with bleeding hardly adhered onto VWF under flow compared to patients with no bleeding symptoms (Figure 2A-C).

This effect appeared reversible as shown in one patient (#03) who interrupted ibrutinib therapy due to infection complication. The recovery of collagen-induced platelet aggregation paralleled the expected physiological platelet renewal rate with nearly half-maximal and complete aggregation restored 60 and 168 h after treatment interruption, respectively (Figure S3B in supplemental appendix).

**Clinical implication**

Our results complement two previous short notes and show that, in vitro, ibrutinib specifically affects GPVI and GPIb-mediated platelet functions. Besides Btk, ibrutinib can also inhibit Tec (in vitro IC50=78 nM) suggesting that inhibition of these two important kinases downstream of these platelet receptors, may be responsible for the observed effect. X-linked agammaglobulinemia patients, deficient in Btk, exhibit only a weak platelet aggregation defect in response to low doses of collagen and no bleeding phenotype, probably because Tec compensates the lack of functional Btk. The concentration of ibrutinib required to inhibit 50% of collagen-induced-platelet aggregation in PRP in our study approximates the peak concentration of the drug in humans. Polymorphisms or drug interactions on ibrutinib metabolism leading to pharmacokinetics or pharmacodynamic variations as well as redundant platelet signaling pathways and variations in GPVI and GPIb expression in patients may contribute to explain that only a subset of treated patients display spontaneous bleeding. The combined action of ibrutinib on GPVI and GPIb pathways likely
explains the defect in primary haemostasis, particularly the bleeding in the microvasculature where the shear rate is elevated. Consistent with this, a recent study confirms the occurrence of mild bleeding episodes in 44% of ibrutinib treated CLL patients.\textsuperscript{20} Our study also suggests that platelet transfusion at a dose sufficient to get 50\% of fresh platelets may correct haemostasis in emergency, provided it is given after elimination of ibrutinib from blood, which takes several hours after last intake.\textsuperscript{6} By analogy, treatment interruption for 5 days may be sufficient before an invasive procedure at high bleeding risk.

Although one patient with reduced aggregation to collagen had no bleeding manifestation, this test seems to be indicative of an increased bleeding risk in patients under ibrutinib treatment as also suggested by a very recent clinical study.\textsuperscript{21} Further prospective studies will determine whether it is useful for guiding the therapeutic strategy, especially for patients under antiplatelet therapy.

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AUTHORSHIP CONTRIBUTION STATEMENT

M.L., E.D., C.G., P-A.L. designed and performed most experiments and analyzed data, S.C. performed flow cytometric analysis, J-C.B. contributed to platelet
aggregation tests, A-S.M. and L.Y. selected patients, M.L., C.T., P.S., L.Y. and B.P. designed research, supervised the work, analyzed data and wrote the paper.

**CONFLICT-OF-INTEREST DISCLOSURE**

The authors declare no competing financial interest.

**References**


FIGURE LEGENDS

Figure 1: Effect of ibrutinib on platelet responses to collagen, CRP and VWF in vitro. (A) Washed platelets from healthy donors were treated or not with ibrutinib at the indicated concentration for 10 minutes and stimulated with different agonists (collagen 3.3 μg/ml, CRP 9 μg/ml, U46619 5 μM, TRAP 50 μM, thrombin 0.5 UI/ml). Platelet aggregation was assessed by turbidimetry and results, expressed as percentage of aggregation, are means ± SEM (collagen: n=12; CRP and other agonists: n=6). In parallel to aggregation, the effect of ibrutinib on platelet signaling in response to 1 minute stimulation with collagen (B,C) or CRP (D) was assessed by western blotting (whole platelet tyrosine phosphorylation pattern, PLCγ2 phosphorylation on Tyr-753 and Src phosphorylation on Tyr-416) or by flow cytometry for Btk phosphorylation on Tyr-223. The insert shows the fluorescence intensity (MFI) in resting (a), CRP-stimulated (b) and CRP-stimulated ibrutinib-treated (c) platelets. Westernblots shown are representative of 3 independent experiments. Results of western blot quantification are means ± SEM of 3 to 6 independent experiments. (E) Effect of increasing doses of ibrutinib on collagen-induced platelet aggregation in PRP (n=4, mean ± SEM). (F) Effect of ibrutinib on platelet adhesion on VWF under arterial flow conditions (4000 s⁻¹ or 180 dyn/cm²) in whole blood. Under these flow conditions, platelet adhesion was dependent on GPIb as verified by its complete inhibition by a monoclonal GPIb antibody (not shown). Blood from healthy donors was preincubated for 30 minutes with 0.5 μM ibrutinib or DMSO. After 5 minutes of flow, firm platelet adhesion was quantified, after washing with PBS containing Ca²⁺/Mg²⁺ at 4000 s⁻¹ during 1 minute, by measuring the platelet surface coverage values (means ± SEM from 3 independent experiments). *p<0.05 and **p<0.01. Scale bar: 50 μm.
Figure 2: Platelet functions of CLL and CML patients treated by Imbruvica® (ibrutinib). (A) Clinical and biological data were collected before initiation of the treatment (day 0) and after 2 to 4 weeks of treatment (day 15-30). Platelet aggregation induced by collagen (3.3 µg/ml), ADP (5 µM) or U46619 (5 µM) on PRP was assessed as in Figure 1 at day 0 and day 15-30 and expressed as means ± SEM. It is noteworthy that platelet aggregation induced by collagen at day 0 was slightly reduced in the patients group compared to healthy donors (88% ± 6% for healthy donors and 69% ± 7% for patients, p<0.01, n=19 healthy donors). Of note, in the non-bleeding group at day 15-30, patients #06 and #10 had a strong reduction of aggregation. Importantly, patient #06 ($) experienced a severe metrorrhagia at day 50. Platelet adhesion on VWF matrix was assessed for 6 patients (#01, #02, #03, #07, #08 and #14) as in Figure 1 and expressed as the surface coverage. Results are presented as means ± SEM. (B) Example of a patient (P#01) showing platelet dysfunction and bleeding symptoms upon ibrutinib treatment. (C) Example of a patient (P#08) with normal platelet responses and no haemostasis-related adverse effect upon ibrutinib treatment. Of note hyperleukocytosis was not correlated to ex vivo platelet dysfunction. *p<0.05 and **p<0.01. Day 0 (D0), day 15 (D15).
Figure 2

(A) Table showing patient numbers, platelet counts, collagen, ADP, U46619, bleeding symptoms, and CTC grade.

(B) Graphs showing maximal platelet aggregation at d15 with various agonists and adhesion on VWF matrix.

(C) Similar graphs to (B) but with different conditions.

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