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Pharmacological effects of small molecule BCR-ABL tyrosine kinase inhibitors on platelet function

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Nonstandard abbreviations:

ALL: acute lymphoblastic leukemia

ACD: acid citrate dextrose

BCR: breakpoint cluster region

BSA: bovine serum albumin

CML: chronic myelogenous leukemia

CRP-XL: cross-linked collagen-related peptide

DIC: differential interference contrast

GPVI: glycoprotein VI

ITAM: immunoreceptor tyrosine-based activation motif

MFI: mean or median fluorescence intensity

TKI: tyrosine kinase inhibitors

PS: phosphatidylserine

PRP: platelet-rich plasma

PPP: platelet-poor plasma

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ABSTRACT

Tyrosine kinase inhibitors (TKIs) targeting the BCR-ABL fusion protein, such as imatinib (Gleevec), have revolutionized targeted cancer therapies. However, drug resistance and side effects, particularly those affecting hemostasis, continue to pose significant challenges for TKI therapies. As tyrosine kinases serve pivotal roles in platelet hemostatic function, we investigated the potential impact of both established and emerging ABL TKIs on human platelet activities *ex vivo*. Our study included standard-of-care agents (e.g., imatinib and nilotinib), and second-generation ABL inhibitors including ponatinib and bosutinib designed to mitigate drug resistance. Additionally, we explored the effects of allosteric inhibitors targeting the myristoyl pocket of ABL (e.g., asciminib and GNF-2), and novel agents in preclinical development, including ELVN-919, which uniquely exhibits high specificity for the ABL kinase active site. Our findings reveal that while ABL inhibitors such as ponatinib and bosutinib impede platelet activity, highly specific new-generation ABL inhibitors, including first-in-class therapeutics, do not impact platelet function *ex vivo*. Overall, these new insights around the effects of ABL TKIs on platelet function could inform the development of targeted therapies with reduced hematologic toxicities.

Significance Statement

This study examines the effects of clinically relevant small molecule BCR-ABL tyrosine kinase inhibitors (TKIs) on platelet activity. This analysis includes first-time assessments of agents such as asciminib and ELVN-919 on human platelet function *ex vivo*, alongside established therapies (e.g., imatinib, ponatinib) with well-characterized effects on platelet function, to discern potential anti-platelet and other effects of BCR-ABL TKIs and inform clinical safety.

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INTRODUCTION

More than 95% of chronic myeloid leukemia (CML) and 20-30% of acute lymphoblastic leukemia (ALL) patients express of the fusion protein BCR-ABL1, following the reciprocal translocation of the long arms of chromosomes 22 and 9, named the Philadelphia chromosome (Burmeister et al., 2008; Eden and Coviello, 2023). The prevalence of this oncoprotein underscores its central role in CML pathogenesis. Translation oef BCR-ABL1 results in the synthesis of a protein exhibiting heightened Abl tyrosine kinase activity correlated with identifiable defects in CML, notably a substantial augmentation in myeloid cell counts. This phenomenon arises from a combination of enhanced cellular proliferation and diminished apoptosis within the hematopoietic stem cell or progenitor cell population (Druker, 2004).

Imatinib (Gleevec), developed as a first-generation ABL tyrosine kinase inhibitor (TKI), has significantly ameliorated the prognosis of patients with CML. As a therapeutic agent, this TKI enhances overall patient survival (Bower et al., 2016); however, approximately 20% of individuals receiving imatinib fail to achieve a complete cytogenetic response (Hoffmann et al., 2017). Consequently, second-generation BCR-ABL inhibitors, namely dasatinib, nilotinib, bosutinib, and radotinib were rationally designed to exhibit greater selectivity against the BCR-ABL tyrosine kinase compared to imatinib and were subsequently approved for clinical use (Eskazan and Keskin, 2017; Rossari et al., 2018). Notably, patients who exhibit T315I mutation on the BCR-ABL fusion protein frequently experience resistance to even these second-generation TKIs, where subsequent generation TKIs, such as ponatinib, can overcome this additional therapeutic challenge (Nicolini et al., 2017). However, side effects, including vascular and pulmonary toxicity, were observed in patients utilizing next-generation TKIs (Moslehi and Deininger, 2015; Valent et al., 2015). In response to this, asciminib, as an allosteric inhibitor, was

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designed with hopes of avoiding these side effects, and recently gaining approval from the FDA. Asciminib achieves deep and stable responses, even in heavily treated patients and those that express the T315I mutation, albeit at significantly higher doses. Nevertheless, a notable proportion of patients still experience intolerance or refractory responses (Scalzulli et al., 2023). It remains to be seen if these side effects can be avoided using mechanistically-distinct inhibitors of BCR-ABL still in preclinical development, including GNF-2, rebastinib, bafetinib, tozasertib, danusertib, and HG-7-85-01 (Rossari et al., 2018).

Tyrosine kinases serve critical roles in orchestrating the signaling pathways that mediate hematopoietic cell activity (Colicelli, 2010), including the hemostatic function of platelets (Zheng et al., 2022). Accordingly, BCR-ABL inhibitors may drive a range of platelet-related side effects around blood clotting and bleeding (Scalzulli et al., 2023). While several studies have individually evaluated the anti-platelet effects of select BCR-ABL kinase inhibitors both *in vitro* and *in vivo* (O'Hare et al., 2009; Loren et al., 2015; Cortes et al., 2018; Scalzulli et al., 2023), comprehensive evaluations of the different generation BCR-ABL inhibitors on platelet function have not yet been reported. Here, we investigated the effects of a range of standard-of-care and more novel pre-clinical ABL inhibitors on platelet adhesion, spreading, activation, aggregation, signaling, and fibrin generation.

MATERIALS AND METHODS

Reagents

Prostacyclin was obtained from Cayman Chemical (Ann Arbor, MI, US), crosslinked collagen-related peptide (CRP-XL) was from R. Farndale (CambCol Laboratories, Cambridge University, Cambridge, UK), Ticagrelor was obtained from Oxchem Corporation (Wood Dale, IL, US), human fibrinogen was

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from Enzyme Research (South Bend, IN, US), collagen was from Chrono-Log (Havertown, PA, US). Bovine thrombin, fatty acid-free bovine serum albumin (BSA), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or as previously mentioned.

ABL Inhibitors

Imatinib, Nilotinib, Ponatinib, Asciminib, Bosutinib, Radotinib, Rebastinib, and GNF-2 were from Selleck Chemicals (Houston, TX, US). ELVN-919, a small molecule Abl kinase inhibitor (Supplemental Fig. S1) was synthesized by Enliven Therapeutics, Inc., as described (Lyssikatos et al., 2022). The inhibitory effects of ELVN-919 on Abl kinase are detailed in the Supplemental Materials and Methods section.

Antibodies

Anti-phosphotyrosine antibody 4G10 (#05-321) was from Millipore (Burlington, MA, US) and α-tubulin antibody (#T6199) was obtained from Sigma (St. Louis, MO, US). Primary antisera against phospho-Src (#2101), Phospho-PKCdelta (#2055), phospho-p38 (#4511), PKC substrates (#2261), Akt substrates (#9614S), phospho-GSK3A (#9316), and phospho-MLC2 (#95777) were obtained from Cell Signaling Technology (Danvers, MA, US). Flow cytometry antibodies APC anti-human CD62P (#304910) and FITC anti-human CD41 (#303704) were obtained from BioLegend (San Diego, CA, US), FITC mouse anti-human PAC-1 (#340507) and APC mouse anti-human CD45 (#506973) were obtained from BD Biosciences (Franklin Lakes, NJ, US), FITC bovine lactadherin was obtained from Haematologic Technologies (Essex Junction, VT, US), and TRITC phalloidin (#P1951) was obtained from Sigma (St. Louis, MO, US).

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Platelet Preparation

Human venous blood was drawn from healthy adult male and female volunteers into sodium citrate (3.8% w/v) and warmed 1:10 acid citrate dextrose (ACD), following a protocol approved by the Institutional Review Board of Oregon Health & Science University, as previously described (Zheng et al., 2021). Anti-coagulated blood was centrifuged at 200 *g* for 20 min to isolate and obtain the platelet rich plasma (PRP). The PRP was then centrifuged at 1000 g for 10 min in the presence of prostacyclin (0.1 μg/mL) and the platelet-poor plasma (PPP) was decanted to isolate a platelet pellet. The collected platelets were re-suspended in modified HEPES/Tyrode (H-T) buffer (129 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) and ACD to the desired concentration for experimental use.

Static Adhesion Assays

For platelet spreading experiments on fibrinogen or collagen, 12-mm no. 1.5 glass coverslips (Fisher Scientific) were coated with human fibrinogen (50 μg/ml) or soluble collagen (50 μg/ml), respectively. The coated surfaces were then treated and blocked with filtered, denatured fatty acid-free BSA (5 mg/ml). Platelets (2×10^7/mL) were pre-incubated with select ABL kinase inhibitors (1 μM) or vehicle (0.1% DMSO) for 10 min. The inhibitor-treated platelets were then seeded onto the immobilized fibrinogen- or collagen-coated coverslip surfaces and incubated at 37 °C for 30 min. The glass coverslips were then washed three times with phosphate-buffered saline (PBS) to remove non-adherent platelets. Adherent platelets were fixed in 4% paraformaldehyde (PFA) for 10 min and washed three times with PBS. Coverslips were mounted on pre-cleaned microscope slides (25×75×1 mm) using

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Fluoromount G (Southern Biotech). Platelets were imaged using Kohler-illuminated Nomarski differential interference contrast (DIC) optics with a Zeiss 63× oil immersion 1.40 numerical aperture (NA) plan-apochromatic lens on a Zeiss Axio Imager M2 microscope using Slidebook 5.5 image acquisition software (Intelligent Imaging Innovations, Denver, CO), as previously described. Three images for each treatment condition were taken. The number of platelets adherent to collagen and individual platelet surface areas on fibrinogen per field of view (14,587 μm^2) were measured and analyzed using Image J (NIH). Data is shown as mean ± SEM; statistical analysis were conducted using a one-way ANOVA test on GraphPad PRISM, where a P value < 0.05 was considered significant.

Measurement of filamentous actin levels

Washed platelets (2×10^8/mL) were pre-treated with ABL kinase inhibitors or vehicle (0.1% DMSO) for 10 min followed by stimulation with the GPVI-specific agonist, CRP-XL (10 μg/mL), for 30 min. The platelets were then fixed with an equal volume of 4% paraformaldehyde for 45 min followed by permeabilization and staining with 0.1% Triton X-100 containing TRITC-phalloidin (1:100) for 60 min. Filamentous actin (F-actin) levels were determined via flow cytometry (BD FACSCanto II flow cytometer, BD Biosciences, Franklin Lakes, NJ, US) and analyses were performed with FlowJo software (version 10.8.1). Platelets were identified by logarithmic signal amplification for forward and side scatter characteristics and mean fluorescence intensity of 10,000 cells quantitated per sample. Data is shown as mean ± SEM; statistical analysis were conducted using a one-way ANOVA test on GraphPad PRISM, where a P value < 0.05 was considered significant.

Platelet Aggregation

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Platelet aggregation studies were performed using 300 µL of platelets (3×10^8/mL) pre-incubated with ABL inhibitors (1 µM), or vehicle (0.1% DMSO) for 10 min in glass cuvettes. Each platelet mixture was stimulated with CRP-XL (2 µg/mL) and monitored at 37°C under continuous stirring at 1200 rpm. The change in light transmission was measured using a Chrono-Log 490 4+4 aggregometer for 4 min (Chrono-Log Corporation, Havertown, PA, USA). A representative trace of three different experiments was presented.

Flow Cytometry and Analysis

Washed, purified platelets were prepared to a concentration of 2×10^8/ml and incubated with the selected ABL inhibitors (1µM) or vehicle (0.1% DMSO) for 10 mins. FITC PAC-1 (1:50) and APC CD62P (1:50), or FITC bovine lactadherin (1:20) were added to stain for activated integrin $\alpha_{IIb}\beta_3$, P-selectin, and phosphatidylserine respectively. Each platelet mixture was stimulated with CRP-XL (10 µg/ml) for 20 min and the reactions were stopped by 2% paraformaldehyde. Untreated platelets (vehicle, 0.1% DMSO) unstimulated and stimulated with CRP-XL (10 µg/ml) served as the negative and positive controls, respectively. Each sample was analyzed using flow cytometry on a BD FACSCantoII flow cytometer (BD Biosciences, Franklin Lakes, NJ, US) and data analyses were performed with FlowJo software (version 10.8.1). Platelet populations were identified and gated by logarithmic signaling amplification for forward and side scatter, as previously described (Aslan *et al.*, 2013). Statistical analysis was performed using a one-way ANOVA test on GraphPad PRISM, where a P value < 0.05 was considered significant.

Fibrin Generation Assay

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50 µL of Washed platelets (5×10^7/mL) were incubated with select ABL kinase inhibitors (1 µM) or vehicle (0.1% DMSO) for 10 min in a 96-well plate. Platelet activation was induced by CRP-XL (10 µg/mL) for 30 min. Citrated platelet-poor plasma (50 µL) and $CaCl_2$ (50 µL, 8.3mM final) were added to each platelet mixture. Fibrin generation was quantified by measuring the changes in turbidity at an absorbance of 405 nm at 30 sec intervals for 90 min using an Infinite M200 spectrophotometer (Tecan, Männedorf, Switzerland). The lag time and time to reach half of maximum turbidity were measured. Statistical analysis was performed using a one-way ANOVA test on GraphPad PRISM, where a P value < 0.05 was considered significant.

Western Blotting

Washed platelets (5×10^8/ml) were incubated with ABL inhibitors (1 µm) or vehicle (0.1% DMSO) for 10 min prior to stimulation with CRP-XL (10 µg/ml, 5 min). The platelet samples were directly lysed into Laemmli Sample Buffer with dithiothreitol (DTT, 200 mM), and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane and analyzed with indicated antibodies by Western blot using a ProteinSimple FluorChem Imaging system.

RESULTS

Effects of BCR-ABL Inhibitors on Platelet Substrate Adhesion

To gain insight into whether BCR-ABL TKIs affect platelet function, we first examined the effects of ABL inhibitors on the ability of platelets to adhere to and spread on fibrinogen-coated surfaces. As seen in Figure 1A, washed platelets prepared from healthy human donors readily adhered to fibrinogen, spreading to a surface area of 32.3±1.3 µm² per platelet on fibrinogen. Pre-treatment of platelets with either ponatinib or bosutinib reduced the platelet surface area on fibrinogen to 16.7±1.0 or 23.9±0.4

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μm², respectively. Platelet spreading on fibrinogen was not significantly affected by pre-treatment with other BCR-ABL inhibitors (Fig. 1B), including imatinib, nilotinib, asciminib, radotinib, rebastinib, GNF-2, or ELVN-919, a 7-azaindole compound which targets the active site of Abl to limit BCR-ABL activity (Lyssikatos et al., 2022).

We next studied the effects of BCR-ABL inhibitors on platelet adhesion on soluble collagen. In the absence of BCR-ABL inhibitors, 71±3 platelets bound to collagen-coated surfaces per each field of view of 14,587 μm^2, while the number of platelets binding to collagen was reduced in the presence of ponatinib or bosutinib (Fig. 1C). Other BCR-ABL inhibitors did not significantly affect the number of adherent platelets on collagen. Next, the effect of BCR-ABL inhibitors on platelet cytoskeletal reorganization downstream of GPVI activation was verified by quantifying TRITC-phalloidin staining of filamentous actin content in platelets stimulated with CRP-XL. As seen in Fig. 1D, pretreatment of platelets with ponatinib or bosutinib reduced F-actin nucleation following CRP-XL stimulation (P=0.029 and P=0.012, respectively), while F-actin levels were unaffected by other BCR-ABL inhibitors, including imatinib, nilotinib, asciminib, radotinib, rebastinib, GNF-2, or ELVN-919.

Effects of BCR-ABL Inhibitors on Platelet Tyrosine Kinase Signaling

Next, we examined the effect of BCR-ABL kinase inhibitors on intracellular signaling cascades downstream of the platelet immunoreceptor tyrosine-based activation motif (ITAM) receptor, GPVI. As seen in Fig. 2, stimulation of platelets with the GPVI-specific agonist crosslinked collagen-related peptide (CRP-XL) promoted the phosphorylation of tyrosine kinase, Akt, and PKC substrates, as well as Src, Lyn, LAT, vasodilator-stimulated phosphoprotein (VASP) and myosin light chain (MLC). Pretreatment of platelets with bosutinib abrogated these ITAM-mediated signaling events downstream of GPVI, including phosphorylation of Src Tyr419, Lyn Tyr507, LAT Tyr220, VASP Ser157 and MLC2

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Thr18+Ser19. Pretreatment of platelets with ponatinib also reduced GPVI-mediated phosphorylation of Akt substrates, VASP, and MLC2. Conversely, pretreatment of platelets with rebastinib upregulated tyrosine kinase signaling while inhibiting Lyn phosphorylation, where c-Cbl serves as a negative regulator of tyrosine phosphorylation downsteam of Lyn activation (Auger et al., 2003). Radotinib treatment also markedly inhibited VASP phosphorylation. Markers of platelet ITAM signaling downstream of GPVI remained unaffected by the remainder of ABL inhibitors including imatinib, nilotinib, asciminib, GNF-2, and ELVN-919. Altogether, our data indicates that ponatinib and bosutinib inhibit signaling downstream of platelet GPVI, while allosteric inhibitors (asciminib and GNF-2), as well as novel agents ELVN-919, do not have a significant impact on these pathways.

Effects of BCR-ABL Inhibitors on Platelet Granule Secretion and Integrin Activation

The granule content stored within platelets plays requisite or at times pathological roles in processes spanning from inflammation, atherosclerosis, wound healing, angiogenesis, and malignancy. Upon GPVI activation, platelets secrete the contents of their α-granules, including the surface translocation of P-selectin (CD62P) (Blair and Flaumenhaft, 2009). Next, we implemented a flow cytometry approach to quantify changes in platelet P-selectin surface expression following platelet activation with CRP-XL. As seen in Fig. 3A, resting and activated washed human platelets (and inhibitor treated platelets, not shown) had similar forward scatter (FSC) and side scatter (SSC) profiles. Stimulation with CRP-XL increased P-selectin on the platelet surface, reported as the mean or median fluorescence intensity (MFI) measured by flow cytometry (Fig. 3B). Preincubation of platelets with ponatinib (1 μM) or bosutinib (1 μM) significantly reduced P-selectin surface expression, whereas pretreatment of platelets with other BCR-ABL inhibitors (imatinib, nilotinib, asciminib, radotinib, rebastinib, GNF-2, and ELVN919) did not significantly affect platelet α-granule secretion following CRP-XL stimulation.

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Following GPVI activation, downstream signaling pathways also support the "inside-out" activation of the platelet surface integrin $\alpha_{IIb}\beta_3$, promoting fibrinogen binding and subsequent platelet-platelet aggregation. Next, we quantified the activation state of platelet surface integrin $\alpha_{IIb}\beta_3$ with flow cytometry using FITC-PAC-1, which specifically reports the active conformation of human integrin $\alpha_{IIb}\beta_3$ (Zheng *et al.*, 2021). As shown in Fig. 3C, PAC-1 binding to platelets increased following stimulation with CRP-XL. Similar to the results for P-selectin above, both ponatinib (1 μM) and bosutinib (1 μM) inhibited platelet PAC-1 binding following stimulation with CRP-XL. Pretreatment of platelets with other BCR-ABL inhibitors did not impact the activation state of platelet integrin $\alpha_{IIb}\beta_3$ after CRP-XL stimulation. In parallel experiments with Born aggregometry, platelet aggregation induced by CRP-XL was likewise abrogated by ponatinib (1 μM) and reduced by bosutinib (1 μM), but largely insensitive to the treatment of platelets with the other BCR-ABL inhibitors (Fig. 4).

Effects of BCR-ABL Inhibitors on Platelet Procoagulant Activity

Later in their activation programs, platelets externalize phosphatidylserine (PS) on the outer leaflet of their plasma membrane to provide a platform for the assembly of coagulation factors to promote thrombin generation (Reddy and Rand, 2020). Next, we used flow cytometry to measure the binding of FITC-conjugated lactadherin to platelets as means to follow platelet PS exposure (Shi et al., 2008; Zheng et al., 2023). As seen in Fig. 5A, pretreatment of platelets with ponatinib significantly decreased the fluorescence intensity of PS exposure induced by CRP-XL. Additionally, ponatinib and bosutinib – and, to a lesser extent, as GNF-2 and ELVN-919 – significantly reduced the percentages of PS-positive platelets detected by flow cytometry following CRP-XL stimulation. Finally, we assessed the effects of BCR-ABL inhibitors on platelet procoagulant activity by quantifying platelet-mediated fibrin generation

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in plasma using light absorbance turbidity assays (Fig. 5C, D). Platelets were preincubated with vehicle alone or inhibitors and subsequently stimulated with CRP-XL (1 μg/mL). Citrated platelet-poor plasma (PPP) and $CaCl_2$ (25 mM) were sequentially introduced to each platelet mixture in equal proportions, and fibrin formation was quantified by measuring the change in turbidity at an absorbance of 405 nm for 2 hours. Under control conditions, fibrin generation began after a lag time of 1230 seconds (±89 seconds) following stimulation with CRP-XL. This lag time was prolonged by the pretreatment of platelets with ponatinib (1 μM), where fibrin generation occured after 1860 seconds (±196 seconds). However, pretreatment with bosutinib (1 μM) did not significantly alter lag time ($P = 0.934$). Consistent with other the above studies, imatinib, nilotinib, asciminib, radotinib, rebastinib, GNF-2, and ELVN-919 did not affect the kinetics of fibrin generation.

DISCUSSION

In this study, we assessed the effects of nine different clinically relevant, small molecule BCR-ABL inhibitors on human platelet function. Current hypotheses regarding bleeding tendencies in patients undergoing targeted cancer therapies suggest that TKIs, including BCR-ABL inhibitors, may impair platelet function (Quintás-Cardama et al., 2009). Conversely, some BCR-ABL inhibitors, including bosutinib and ponatinib have noted cardiovascular and thrombotic complications (Quintás-Cardama et al., 2009; Deb et al., 2020; Brümmendorf et al., 2022). At present, mechanisms underlying these varied clinical observations in patients treated with BCR-ABL inhibitor therapies remain unresolved. Our results demonstrate that, *ex vivo*, both ponatinib and bosutinib reduce platelet adhesion, secretion, integrin activation, aggregation, and ITAM signaling. In contrast, imatinib and newer-generation BCR-ABL inhibitors such as asciminib, GNF-2, and ELVN-919 exhibited no significant effects on platelet function in *ex vivo* conditions tested herein.

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Bosutinib, a dual Src/Abl kinase inhibitor, was initially developed to treat Src-overexpressing solid tumors, yielding positive therapeutic outcomes. Its enhanced selectivity for BCR-ABL compared to imatinib, coupled with a more manageable side effect profile, led to its incorporation into the treatment of CML in 2012. Bosutinib not only inhibits Src/Abl but also induces a significant G1 arrest in clonal hematopoietic progenitors expressing BCR–ABL. This arrest results from a reduction in cyclin-dependent kinase (Cdk) 2 activity, and is linked to changes in the expression or subcellular location of associated regulatory systems around PI3K and Akt signaling (Mancini et al., 2007). Despite these advances, the potential of bosutinib to act as a platelet Src inhibitor and antagonist has remained unexplored. In this study, we observed that *in vitro*, bosutinib markedly diminished overall levels of tyrosine-phosphorylated proteins, including Src, Lyn, and LAT in platelets. Additionally, our data reveals that bosutinib inhibits granule secretion, integrin activation, aggregation, and platelet cytoskeletal rearrangement, potentially through the suppression of MAP4K4 kinase activity (Kleinveld et al., 2021). Recent research indicates the expression of MAP4K4 in human platelets, and inhibiting this site appears to attenuate collagen-, ADP-, and thrombin-induced platelet aggregation, granule release, TXA2 generation, and integrin $\alpha_{IIb}\beta_3$activation (Nam et al., 2021).

Ponatinib, a third-generation BCR-ABL TKI, is utilized in the treatment of CML patients with specific BCR-ABL kinase domain T315I mutations. These mutations confer resistance to imatinib, as well as the second-generation BCR-ABL inhibitors nilotinib, dasatinib, or bosutinib. However, several clinical trials have indicated that, compared to other TKIs, ponatinib promotes adverse thrombotic events (Nazha et al., 2013; Moslehi and Deininger, 2015; Quilot et al., 2016; Barber et al., 2017), thus significantly limiting its clinical utility. Our data shows, *in vitro*, that ponatinib is a platelet antagonist and inhibits platelet activation, granule secretion, spreading, aggregation, and phosphatidylserine exposure through ITAM signaling. This observation suggests that ponatinib-induced vascular adverse events may not be platelets,

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but rather other cell types. For example, previous research has indicated that ponatinib treatment increases endothelial cell dysfunction and apoptosis (Gover-Proaktor et al., 2017), both contributing to a higher incidence of vascular adverse events. Similarly, recent studies propose that ponatinib, by upregulating endothelial ERK5 SUMOylation, induces an inflammatory phenotype in endothelial cells, disrupting vascular homeostasis (Paez-Mayorga et al., 2018). Future work will verify whether ponatinib targets other cell types, potentially contributing to thrombotic events. Moreover, some studies propose that ponatinib's impact on platelet function may be attributed to its potent and broad spectrum of multitargeted activity, particularly on SFKs (Neelakantan et al., 2012). Previous research has demonstrated a substantial reduction in overall levels of tyrosine-phosphorylated proteins and a significant decrease in the activation of Src, phosphorylated Lyn, and LAT in platelets upon ponatinib treatment (Loren et al., 2015).

The ABL protein's kinase core is surrounded by a structure named N-terminal cap (N-cap) region and an extensive stretch of amino acids located at the C-terminal end of the kinase domain (Panjarian et al., 2013). Additionally, the N-cap undergoes myristoylation in the 1b splice variant of the Abl protein. This myristic acid group at the N-terminus forms a bond with a deep hydrophobic pocket situated in the C-terminal lobe (C-lobe) of the kinase domain. This interaction between the myristoylated N-cap and the C-lobe plays a critical role in preserving the autoinhibited state (Pluk et al., 2002; Nagar et al., 2003). In more than 95% of chronic myeloid leukemia (CML) cases, patients exhibit the fusion oncoprotein BCR-ABL (Burmeister et al., 2008; Eden and Coviello, 2023). Ultimately, changes to the Abl N-terminus contribute to heightened cellular proliferation and reduced apoptosis within the hematopoietic stem cell or progenitor cell population, manifesting as pathological features in CML (Panjarian et al., 2013). Unlike other TKIs, asciminib and GNF-2 function as allosteric inhibitors, engaging a vacant pocket at a site within the kinase domain typically occupied by the myristoylated N-

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terminal of ABL (Zhang et al., 2010; Hughes et al., 2019). This unique mechanism enables these drugs to inhibit the activity of BCR-ABL fused proteins in the presence of mutations in the ATP-binding site, including T315I (Hughes et al., 2019). In our study, asciminib, GNF-2, and ELVN-919 demonstrated no significant impact on the activation, granule secretion, spreading, aggregation, and phosphatidylserine exposure of platelets *in vitro* following activation of platelet GPVI. This suggests that this particular mechanism may not interfere with platelet function through ITAM signaling. However, further work is required to determine whether these compounds influence other platelet pathways, such as PAR1, PAR4, and others. Additional studies are also needed to specify the multifacted effects of Abl inhibitors on platelets, other blood cells and vascular cells, where agents such as ponatinib can have procoagulant effects on endothelial cells and have been associated with thrombosis *in vivo*, despite *ex vivo* inhibitory effects on platelets (Gover-Proaktor et al., 2019; Hamadi et al., 2019; Latifi et al., 2019).

In conclusion, our study examines multiple aspects of BCR-ABL tyrosine kinase inhibitors on platelet function. Through *in vitro* assessments, we have determined the differential effects of multiple classes of BCR-ABL inhibitors on platelets, noting that while ponatinib and bosutinib exert strong effects on platelet functions, other inhibitors such as imatinib, asciminib manifest only negligible effects under similar conditions. These observations note the complexity inherent in targeting the BCR-ABL oncoprotein, where therapeutic efficacy must be balanced in contexts of potential disruptions to critical platelet function. The insights gained from this study enhance the understanding of drug-induced platelet dysfunction, and also provide a rationale for the development of better targeted and safer therapeutic agents. Altogether, our findings provide valuable insights into the mechanisms of action of BCR-ABL inhibitors on platelets, presenting potential opportunities for the development of drugs aimed at mitigating hematologic toxicity.

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship contributions

Y.Z., J.P.L, S.D.G, and J.E.A. participated in research design; Y.Z., C.Y., A.R.M., K.S., S.D.S., I.P.I and J.P. conducted experiments; Y.Z., C.Y., A.R.M and J.E.A. performed data analysis; J.P.L. and S.D.G. contributed new reagents; Y.Z., J.J.S., T.Z., O.J.T.M., and J.E.A. wrote or contributed to the writing of the manuscript.

Disclosure

J.J.S. serves as a medical consultant for Aronora, Inc. This potential conflict of interest has been reviewed and managed by the Oregon Health & Science University Conflict of Interest in Research Committee. J.P.L. and S.D.G. are employed by Enliven Therapeutics, Inc., a company that may have a commercial interest in the results of this research. The remaining authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1. Effect of BCR-ABL kinase inhibitors on platelet surface spreading. (A) Replicate samples of washed human platelets (2×10^7 mL^{-1}) treated for 10 min with BCR-ABL kinase inhibitors (1 μM) or vehicle (0.1% DMSO) were spread on fibrinogen (50 μg mL^{-1}) or soluble collagen-coated (100 μg mL^{-1}) glass coverslips for 30 min at 37°C. After fixation, the coverslips were mounted on microscope slides (25 × 75 × 1 mm). Adherent platelets were visualized using Nomarski differential interference contrast (DIC) microscopy at × 63,000 magnification. Scale bar = 10 μm. Results are representative of three experiments. (B) Three images of platelets under each BCR-ABL inhibitor (1 μM) treatment were captured, the graph displays the average surface area of platelets adherent to fibrinogen glass coverslips, quantified in Image J. (C) Three images of platelets under each BCR-ABL inhibitor (1 μM) treatment were captured, and the number of platelets adherent to collagen per field of view (14,587 mm^2) were counted, then quantified by Image J. (D) Platelet F-actin levels were analyzed by flow cytometry. Data are shown as mean ± S.E.M.; statistical analysis was conducted using a one-way ANOVA test on GraphPad PRISM. * signifies *P* value < 0.05 with respect to DMSO control. N=3. Ima, imatinib; Nil, nilotinib; Pon, ponatinib; Asc, asciminib; Bos, bosutinib; Rad, radotinib; Reb, rebastinib; GNF, GNF-2; 919, Enliven-919.

Figure 2. Effects of BCR-ABL Inhibitors on GPVI-mediated Platelet Tyrosine Kinase Signaling. Replicate samples of washed human platelets (5×10^8 mL^{-1}), treated for 10 minutes with BCR-ABL inhibitors (1 μM) or vehicle (DMSO, 0.1%), were stimulated with GPVI agonist CRP-XL. After 5 min, platelets were lysed in Laemmli sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and analyzed for tyrosine phosphorylation with 4G10 antisera, Src Tyr419 phosphorylation, Lyn Tyr507 phosphorylation, LAT Tyr220 phosphorylation, vasodilator-stimulated phosphoprotein (VASP), myosin light chain (MLC2) phosphorylation, as well as the phosphorylation of PKC and Akt substrates. α-Tubulin serves as a loading control. Western blots are representative of three experiments. TKI: tyrosin kinase inhibitor; CRP-XL, crosslinked collagen-related peptide.

Figure 3. Effects of BCR-ABL Inhibitors on GPVI-mediated P-selectin and Integrin Activation. Washed platelets (2×10^8 mL^{-1}) were treated with BCR-ABL kinase inhibitors (1 μM) or vehicle (0.1% DMSO), followed by CRP-XL (10 μg mL^{-1}) stimulation and staining with APC-CD62P and FITC-PAC1 to monitor platelet α-granule secretion and integrin activation, respectively. (A) Representative forward (FSC) and side scatter (SSC) plots of washed platelets (control and +CRP-XL) analyzed by flow cytometry. (B) Platelet surface P-selectin levels were analyzed by flow cytometry. (C) Platelet PAC-1 levels were analyzed by flow cytometry. Results are shown as mean fluorescence intensity (MFI). Results are representative of three experiments. Data are shown as mean ± S.E.M.; statistical analysis was conducted using a one-way ANOVA test on GraphPad PRISM. * indicates a significant difference compared to the DMSO group following stimulation with CRP-XL, with a *P* value < 0.05. CRP-XL, crosslinked collagen-related peptide; Ima, imatinib; Nil, nilotinib; Pon, ponatinib; Asc, asciminib; Bos, bosutinib; Rad, radotinib; Reb, rebastinib; GNF, GNF-2; 919, Enliven-919.

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Figure 4. Effects of BCR-ABL Inhibitors on GPVI-mediated Platelet Aggregation. (A) Replicate samples of washed human platelets (2×10^8 mL^{-1}) were treated with BCR-ABL kinase inhibitors (1 μM) or vehicle (0.1% DMSO) for 10 min before stimulation with CRP-XL (2 μg mL^{-1}) and analysis by Born aggregometry. Platelet samples were monitored at 37°C under continuous stirring at 1200 rpm for 4 min. Changes in light transmission were recorded as a vertical drop. (B) Representative traces of platelet samples treated with each inhibitor are shown and quantified. Results are representative of three experiments. Data are shown as mean ± S.E.M.; statistical analysis was conducted using a one-way ANOVA test on GraphPad PRISM. * indicates a significant difference compared to the vehicle group, with *P* value < 0.05. Ima, imatinib; Nil, nilotinib; Pon, ponatinib; Asc, asciminib; Bos, bosutinib; Rad, radotinib; Reb, rebastinib; GNF, GNF-2; 919, Enliven-919.

Figure 5. Effects of BCR-ABL Inhibitors on GPVI-mediated phosphatidylserine exposure and fibrin formation. Washed human platelets (2×10^8 mL^{-1}) were treated with BCR-ABL kinase inhibitors (1 μM) or vehicle (0.1% DMSO), followed by CRP-XL (10 μg mL^{-1}) stimulation and staining with FITC-lactadherin to assess (A) mean fluorescence intensity (MFI) and (B) percentage of phosphatidylserine (PS) positive platelets by flow cytometry. Resting or CRP-XL stimulated platelet samples were incubated with citrated platelet-poor plasma (33% final) and re-calcified with $CaCl_2$ (8.3 mM final). Fibrin generation was measured as a change in turbidity at an absorbance of 405 nm. Representative traces are shown (C), and the lag time was measured (D). Results are representative of three experiments. Data are shown as mean ± S.E.M.; statistical analysis was conducted using a one-way ANOVA test on GraphPad PRISM. * indicates a significant difference compared to the 0.1% DMSO group following stimulation with CRP-XL, with *P* value < 0.05. CRP-XL, crosslinked collagen-related peptide; Ima, imatinib; Nil, nilotinib; Pon, ponatinib; Asc, asciminib; Bos, bosutinib; Rad, radotinib; Reb, rebastinib; GNF, GNF-2; 919, Enliven-919.

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Figure 1; Zhang et al.

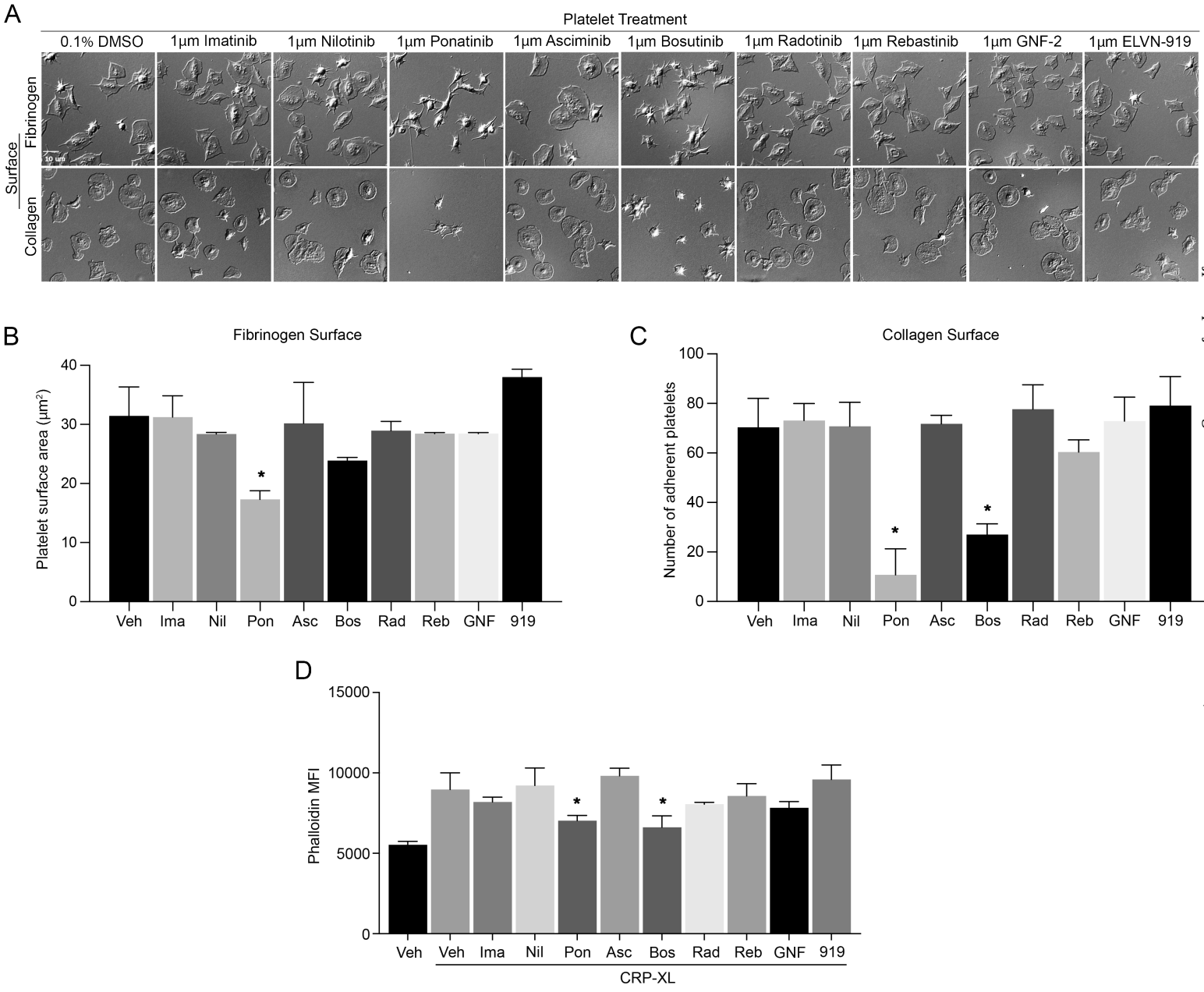

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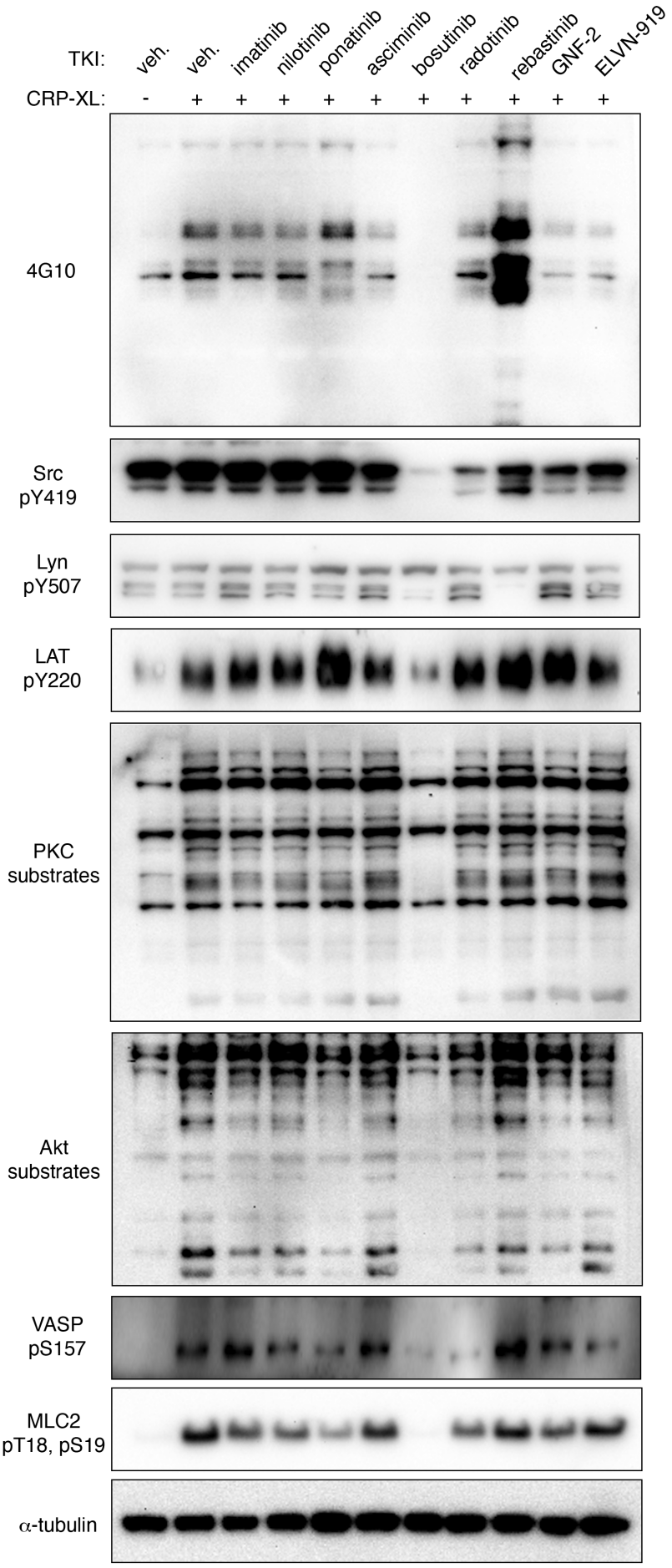
Figure 2; Zhang et al.



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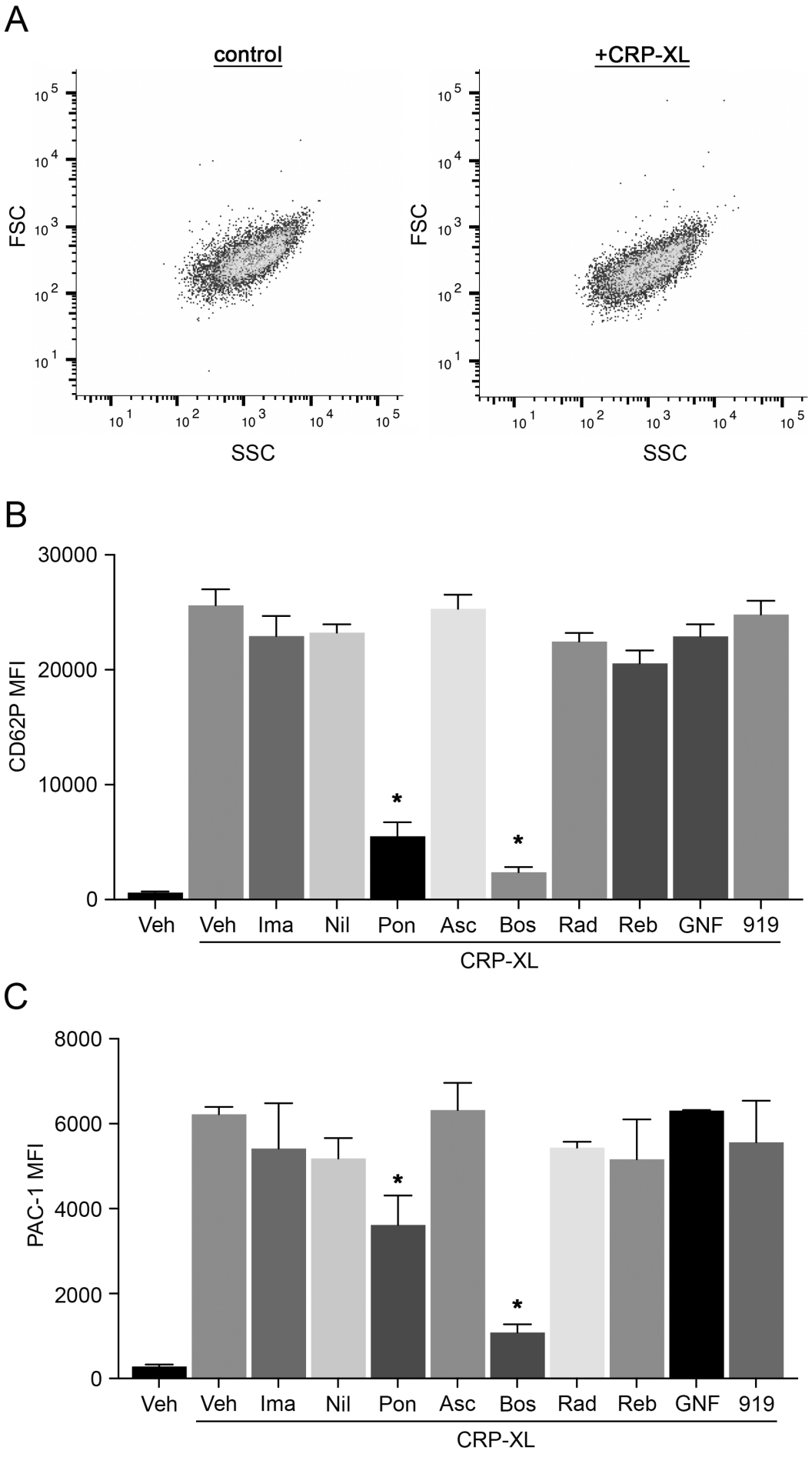

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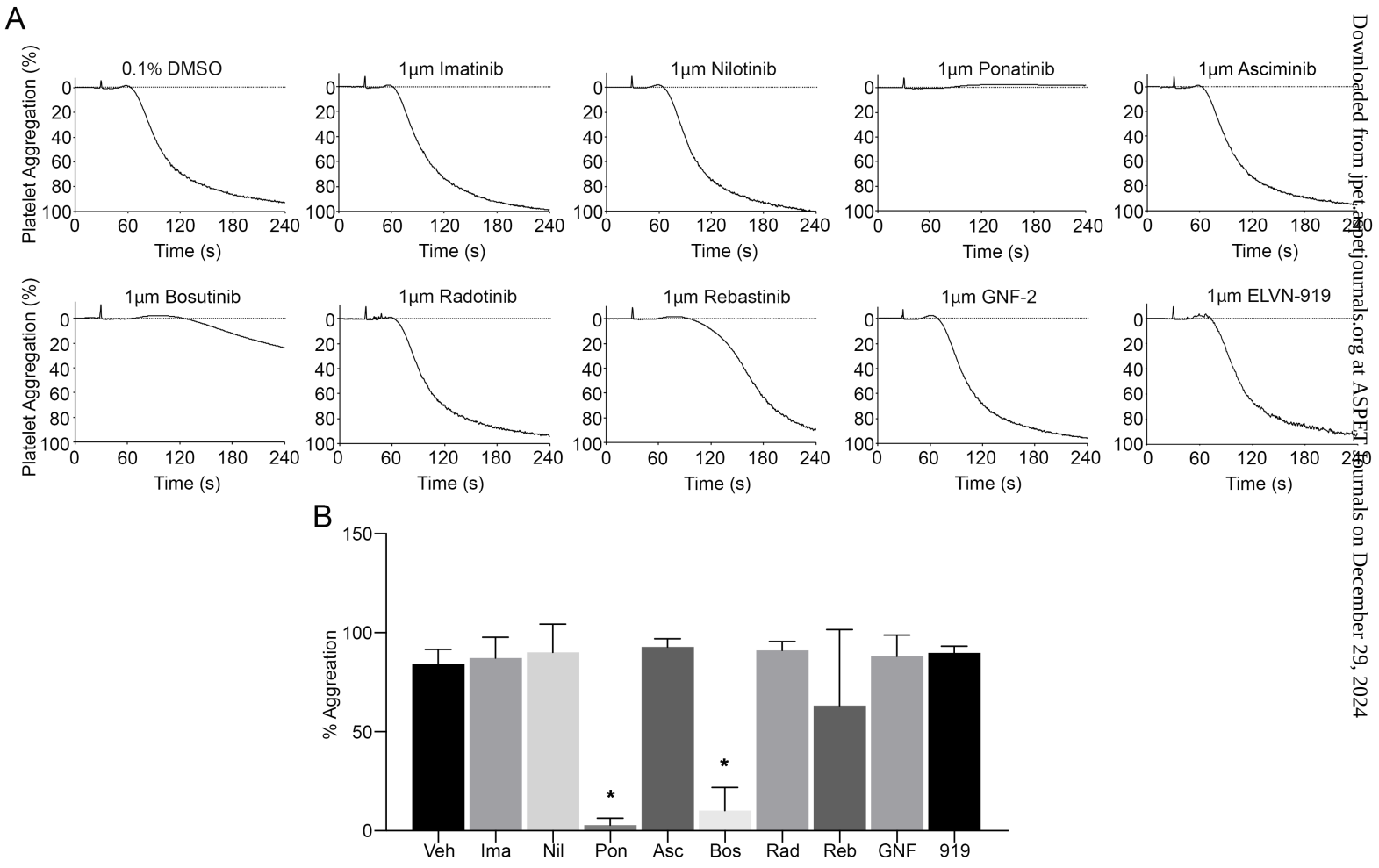
Figure 4; Zhang et al.



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