Impact of Tyrosine Kinase Inhibitors Applied for First-Line Chronic Myeloid Leukemia Treatment on Platelet Function in Whole Blood of Healthy Volunteers In Vitro

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Abstract

The tyrosine kinase inhibitors (TKIs) imatinib, dasatinib, bosutinib, and nilotinib are established for first-line treatment of chronic myeloid leukemia (CML) but may cause side effects such as bleeding and thrombotic complications. We investigated the impact of TKIs on platelet function ex vivo in anticoagulated whole blood (WB) samples from healthy adults by lumiaggregometry and PFA-100 test. Samples ($n = 15$ per TKI) were incubated for 30 minutes with TKI at therapeutically relevant final concentrations. Aggregation and ATP release were induced by collagen (1 µg/mL), arachidonic acid (0.5 mmol/L), and thrombin (0.5 U/mL). Imatinib, bosutinib, and nilotinib significantly increased collagen-induced aggregation compared with controls. In addition, for bosutinib and nilotinib, a significant increase in aggregation after induction with arachidonic acid was detected. ATP-release and PFA-100 closure times were not influenced significantly by these three TKI. In contrast, dasatinib demonstrated a concentration-dependent inhibition of collagen-induced aggregation and ATP release and a significant prolongation of the PFA-100 closure time with the collagen/epinephrine cartridge. Aggregation and ATP release by other agonists as well as closure time with the collagen/ADP cartridge were not influenced significantly. In conclusion, we clearly show a concentration-dependent inhibition of collagen-induced platelet function in WB by dasatinib confirming prior results obtained in platelet-rich plasma. Bosutinib and nilotinib exerted no impairment of platelet activation. On the contrary, both TKI showed signs of platelet activation. When comparing our results with existing data, imatinib in therapeutic relevant concentrations does not impair platelet function.

Keywords

- tyrosine kinase inhibitors
- platelet pathology inherited/acquired
- chronic myeloid leukemia
- BCR-ABL1
- primary hemostasis

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Zusammenfassung

Die Tyrosinkinaseinhibitoren (TKI) Imatinib, Dasatinib, Bosutinib und Nilotinib sind als Erstlinientherapie der chronischen myeloischen Leukämie etabliert, können aber Nebenwirkungen wie Blutungen und thrombotische Komplikationen verursachen. Wir untersuchten den Einfluss dieser TKI auf die Thrombozytenfunktion ex vivo in antikoagulierten Vollblutproben gesunder Erwachsener mittels Lumiaggregometrie und dem PFA-100®-Test. Die Proben ($n = 15$ pro TKI) wurden für 30 Minuten mit TKI in therapeutisch relevanten Endkonzentrationen inkubiert. Aggregation und ATP-Freisetzung wurden durch Kollagen (1 µg/ml), Arachidonsäure (0,5 mmol/l) und Thrombin (0,5 U/ml) induziert. Für Imatinib, Bosutinib und Nilotinib konnte eine signifikante Steigerung der kollageninduzierten Aggregation im Vergleich zur Kontrolle gezeigt werden. Zusätzlich wurde für Bosutinib und Nilotinib eine signifikante Steigerung der Aggregation nach Arachidonsäure-Induktion gemessen. Die ATP-Freisetzung und die PFA-100®-Verschlusszeiten wurden durch diese drei TKI nicht signifikant beeinflusst. Im Gegensatz dazu zeigte Dasatinib eine konzentrationsabhängige Hemmung von kollageninduzierter Aggregation und ATP-Freisetzung sowie eine signifikante Verlängerung der PFA-100®-Verschlusszeiten mit der Kollagen/Epinephrin-Messzelle. Sowohl die Aggregation und ATP-Freisetzung durch andere Agonisten als auch die Verschlusszeiten der Kollagen/ADP-Messzelle wurden nicht signifikant beeinflusst. Zusammenfassend konnten wir eindeutig eine konzentrationsabhängige Hemmung der kollageninduzierten Thrombozytenfunktion im Vollblut durch Dasatinib zeigen und damit frühere Ergebnisse aus plättchenreichem Plasma bestätigen. Bosutinib und Nilotinib bewirkten keine Einschränkung der Thrombozytenaktivierung. Beide TKI führten im Gegenteil zu einer Thrombozytenaktivierung. Imatinib beeinträchtigt in Zusammenschau mit der verfügbaren Literatur in therapeutisch relevanten Konzentrationen die Plättchenfunktion nicht.

Schlüsselwörter

- Tyrosinkinaseinhibitoren
- Pathologie der Blutplättchen vererbt/erworben
- chronische myeloische Leukämie
- BCR-ABL1
- primäre Hämostase

Introduction

Constitutive activation of the tyrosine kinase BCR-ABL1, an oncogenic fusion protein, is the biochemical hallmark of Philadelphia chromosome-positive (Ph+) leukemias. So far, three generations of tyrosine kinase inhibitors (TKIs), inhibiting BCR-ABL1, are used clinically to treat these malignancies. The first-generation TKI imatinib improved long-term survival in adult and pediatric patients with chronic myeloid leukemia (CML) without stem cell transplantation.[1–3] The second-generation TKIs — nilotinib, dasatinib, and bosutinib — which are now approved as upfront treatment for CML in chronic phase (CML-CP) induce a significantly deeper and faster reduction of the *BCR-ABL1* transcript levels.[4] But, of note, patients with a T315I mutation within the kinase domain of *BCR-ABL1* are resistant to imatinib and all second-generation and TKIs are presently treated with the third-generation TKI ponatinib.[5] As of today, TKIs are used as first, second, or further lines of treatment according to CML disease stage and risk scores present, patient comorbidities, and the presence of *BCR-ABL1* mutations contributing to TKI resistance in adult and pediatric patients.[6] TKI treatment considerably improved the outcome of CML, and patients with CML-CP show a life expectancy comparable to those of healthy individuals.

Mechanistically, all TKIs were designed to bind to the adenosine triphosphate (ATP) binding site of the kinase domain of the BCR-ABL1 protein and, thus, inhibiting its further downstream activating functions. As the ATP binding site is a hallmark of several kinases, TKIs show inhibitory off-target effects on multiple other tyrosine kinases in vitro.[7,8] These off-target effects were verified in vivo in clinical trials[9] and characterize each TKI's target kinase profile, its safety, and efficacy.[10] Among the off-target effects described in vitro and in vivo are alterations of platelet function and vascular endothelium[11,12]; thus, BCR-ABL1-TKIs are associated with both bleeding and thrombotic complications. At diagnosis of CML, the *BCR-ABL1* rearrangement is also present in the megakaryopoietic lineage resulting in dysfunctional megakaryocytes and platelets.[13] In addition, extremely high platelet counts (> 1 million/µL) are typically found in 5 to 15% of CML patients at diagnosis, which may result in acquired von Willebrand disease.[14]

However, it has been demonstrated that imatinib at high doses inhibits adenosine diphosphate (ADP), collagen-related peptide, and collagen-induced platelet aggregation and causes a reduced protease-activated receptor-4 mediated α-granule release.[15] Still, the detailed mechanism by which imatinib inhibits platelet functions is under debate.[16] Nilotinib, an improved derivate of imatinib, acts more selectively, but still shows off-target effects on tyrosine kinases such as platelet-derived growth factor receptor (PDGFR), c-KIT, ARG, EPHB4. It has been shown that nilotinib can potentiate a

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Age ≥18 y	Last meal < 6 h ago
≥7 d since last infectious disease	Suspected or known bleeding disorder
≥7 d since last NSAID intake	Known systemic disease
Non-smoker or ≥3 d since last nicotine use	Intake of coagulation-affecting medication in the past 7 d (except oral contraceptive)

Abbreviation: NSAID, nonsteroidal anti-inflammatory drug.

prothrombotic state.[15] But still, some authors claim that nilotinib has no effect on platelet aggregation and, consequently, it has been used as a negative control in in vitro experiments.[17] The second-generation TKI dasatinib has been developed with the intention of a stronger ABL1 and a broader and more potent multi-kinase inhibitory profile. Besides c-KIT, EPHA2, and PDGFR, additional kinases are inhibited such as SFKs, ephrins, BTK, and Syk. Previous studies with dasatinib have demonstrated an impaired collagen-induced platelet adhesion and aggregation.[18,19] The toxicity profile of bosutinib differs distinctly from the other currently approved second-generation TKI in CML. This difference is due to its structural design and the resulting minimal inhibitory activity against PDGFR and KIT, and a different inhibition profile against other target kinases.[7] The clinical effects of bosutinib on platelet function are only marginal when compared with other TKI.[20] Clinical bleeding events of all grades occurred in only 5% of bosutinib-treated CML patients[21] and 85% patients tested ex vivo exhibited normal platelet aggregation.[15,19,22–24] As the data on TKI effects on platelet function are contradictory and the investigations so far were either focused on platelet-rich plasma, performed at non-therapeutically relevant TKI concentrations or performed only for certain TKI, we present here the effect of the TKIs imatinib, dasatinib, nilotinib, and bosutinib on platelet function in vitro in whole blood from healthy young adults in a comparatively manner.

Methods

Citrated whole blood samples were obtained from 38 healthy medical student volunteers (median age: 23 years, range: 18–33 years, 30 females, 8 males) following the inclusion and exclusion criteria listed in ►Table 1. Informed consent was obtained in accordance with the Local Ethical Board Votum (permit number EK 23012015). In each subject, blood cell counts and von Willebrand factor (vWF)-dependent parameters such as vWF antigen, vWF activity, and collagen-binding activity as well as the blood group were determined.

For each TKI test series, 15 different samples were incubated for 30 minutes with different, therapeutically relevant TKI concentrations as listed in ►Table 2 together with further references on these concentrations. All TKIs were dissolved in DMSO and controls were incubated with DMSO only. Platelet aggregation and ATP release were investigated by impedance aggregometry and luminometry (Model 560 CA; Chrono-Log Corp., Havertown, Pennsylvania, United States) as previously described.[25,26] As inductors, collagen (final concentration: 1 µg/mL), arachidonic acid (0.5 mmol/L), and thrombin (0.5 U/mL) were used. Aggregation curves were evaluated 6 minutes after addition of the inductors for maximal aggregation, lag time, slope, and area under the aggregation curve (AUC). ATP release curves were recorded for at least 2 minutes and the maximal ATP release was quantified using an ATP standard (2 nmol).

In TKI-incubated buffered citrated whole blood samples, function of primary hemostasis was investigated by the PFA-100 test (Siemens Healthcare, Germany) measuring the closure times of the collagen/epinephrine and collagen/ADP cartridge as described previously.[27]

Statistical analysis of data was performed using the Wilcoxon rank sum test. Due to abnormal vWF parameters indicative of vWF syndrome, one of the nilotinib measurements was excluded from statistical analysis.

Results

Dasatinib

Dasatinib (0.4 µmol/L) significantly decreased collagen-induced aggregation compared with controls. In 13 out of the

Table 2 TKI concentrations

TKI	Final concentration	Therapeutic range (C0–Cmax)	Reference
Dasatinib	0.2 and 0.4 µM	0.005–0.370 µM	31,40
Imatinib	1.5 µM	1.00–6.08 µM	30,31
Bosutinib	0.4 µM	0.11–0.39 µM	48,49
Nilotinib	4.0 µM	0.94–11.36 µM	31,50,51

Abbreviation: TKI, tyrosine kinase inhibitor.

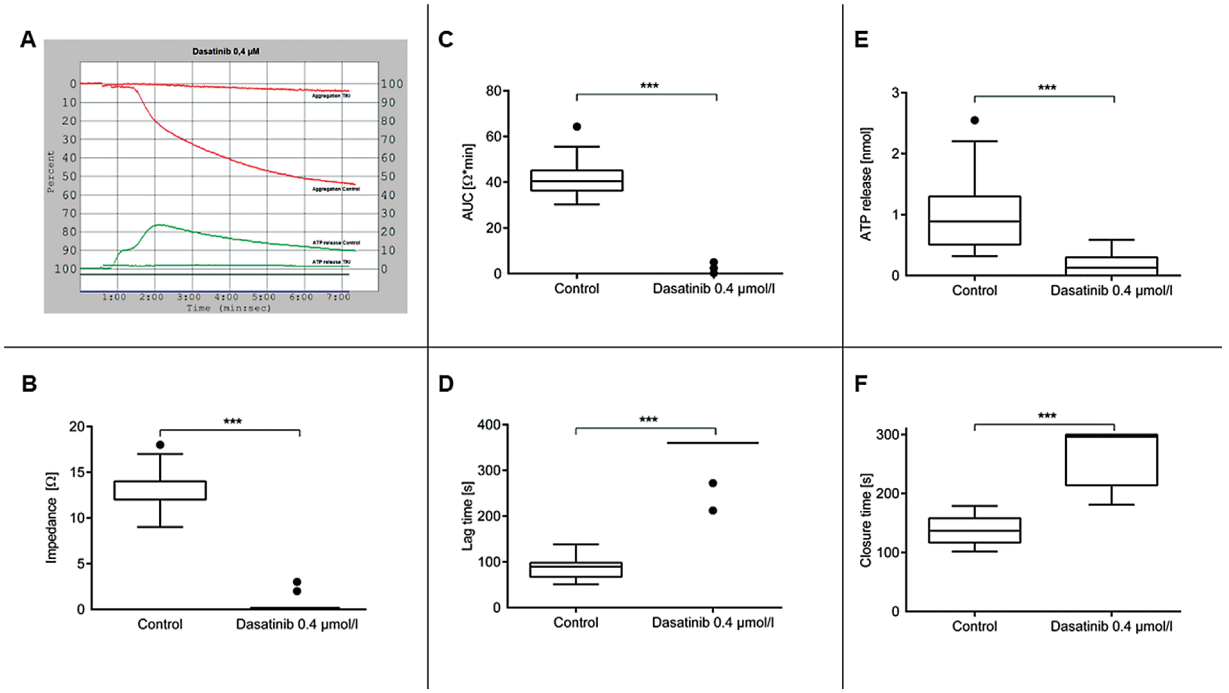

Fig. 1 Panel overview of the main results from measurements with dasatinib at 0.4 µmol/L. (**A**) Representative aggregation (red) and ATP release (green) curves after induction with collagen for a sample incubated with dasatinib at 0.4 µmol/L. (**B**) Collagen-induced maximum platelet aggregation at 6 minutes in samples incubated with dasatinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). Box blots denote the median value and 25/75 interquartile range, while whiskers indicate 1.5 times the interquartile range. Outliers are marked as dots. The boxplot for the dasatinib samples lies on the *x*-axis. (**C**) Area under the curve (AUC) after collagen induction in samples incubated with dasatinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**D**) Lag time after collagen induction in samples incubated with dasatinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**E**) ATP release after collagen induction in samples incubated with dasatinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**F**) Comparison of PFA-100 closure times of the collagen/epinephrine cartridge in samples incubated with dasatinib at 0.4 µmol/L and controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$).

15 samples (87%), no aggregation was detected until 6 minutes after collagen induction of aggregation (►Fig. 1B) causing a significant prolongation of lag time and decrease in AUC (►Fig. 1C) and slope. Reduction of dasatinib concentration by half (final: 0.2 µmol/L) demonstrated no significant changes in the maximum platelet aggregation induced by collagen, but the lag time remained significantly prolonged albeit not as pronounced as with dasatinib at 0.4 µmol/L (►Fig. 2B), which consecutively led to a significant decrease in the measured AUC while the slope was not significantly impacted. Furthermore, ATP release after collagen-induced platelet aggregation was significantly decreased at both dasatinib concentrations, although more pronounced at the higher concentration versus lower concentration (►Fig. 1E). Representative aggregation and ATP release curves for both dasatinib concentrations are shown in ►Figs. 1A and 2A.

Except for the lag time, which was statistically significantly decreased in samples incubated with dasatinib at 0.2 µmol/L, arachidonic acid caused no significant change in any of the aggregation parameters. The thrombin-induced maximum ATP release was also not significantly influenced by both dasatinib concentrations tested.

The PFA-100 closure times of the collagen/epinephrine cartridge were significantly prolonged in samples incubated with 0.4 and 0.2 µmol/L of dasatinib. In 9 out of 15 (60%) and 7 out of 15 (47%) samples, no membrane closure was observed after incubation with dasatinib at 0.4 and 0.2 µmol/L, respectively (►Fig. 1F and 2D). In contrast, closure times of the collagen/ADP cartridge were not significantly different for dasatinib concentrations tested.

Imatinib

Preincubation of samples with imatinib at 1.5 µmol/L led to a significant increase in collagen-induced maximum aggregation and AUC (►Fig. 3) compared with controls, while lag time and slope remained unchanged. Compared with controls, the preincubation of samples with imatinib did influence neither platelet aggregation induced by arachidonic acid nor collagen- and thrombin-induced ATP release. PFA-100 closure times also remained similar for TKI samples and controls.

Bosutinib and Nilotinib

The measurements performed in samples preincubated with bosutinib at 0.4 µmol/L and nilotinib at 4 µmol/L provided comparable results. Both TKIs led to a significantly increased collagen- (►Figs. 4A/B and 5A/B) and arachidonic acid–induced maximum aggregation (►Figs. 4C/D and 5C/D) and AUC. For arachidonic acid induction, the lag time was significantly reduced with both TKIs (►Figs. 4E and 5E). All other aggregation parameters and ATP release induced by collagen and thrombin and the PFA-100 closure times were

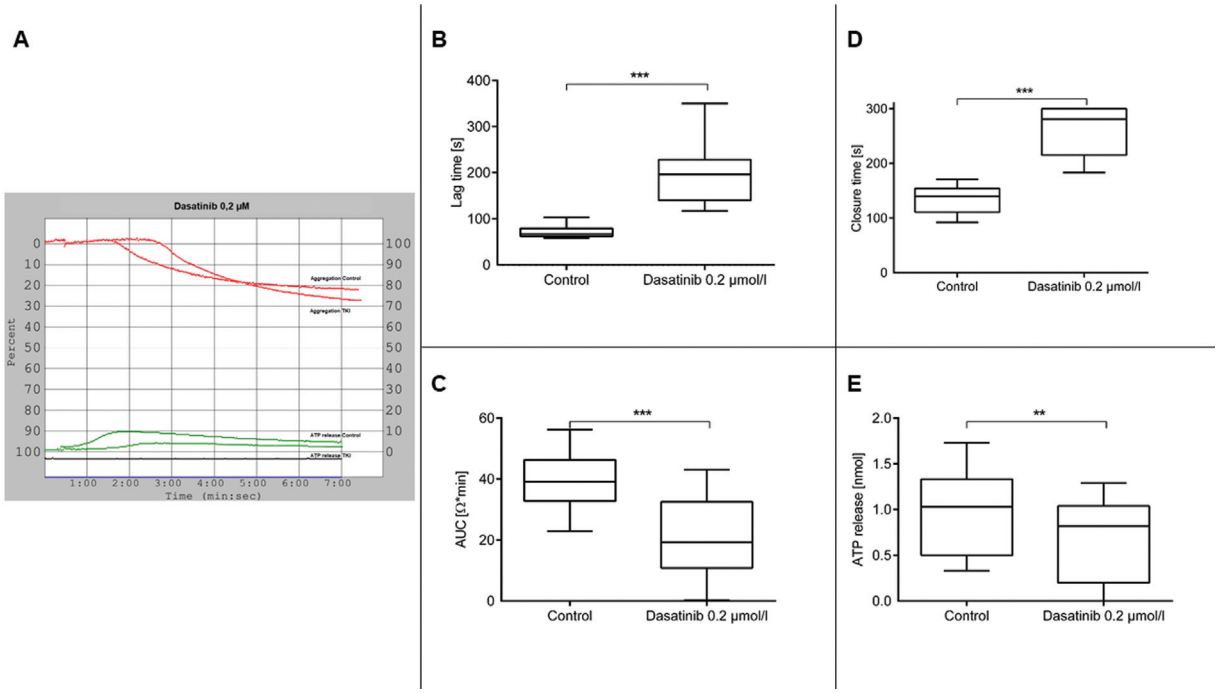

Fig. 2 (**A**) Representative aggregation (red) and ATP release (green) curves after induction with collagen for a sample incubated with dasatinib at 0.2 µmol/L. (**B**) Lag time after collagen induction in samples incubated with dasatinib at 0.2 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**C**) Area under the curve (AUC) after collagen induction in samples incubated with dasatinib at 0.2 µmol/L versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**D**) Comparison of PFA-100 closure times of the collagen/epinephrine cartridge in samples incubated with dasatinib at 0.2 µmol/L and controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**E**) ATP release after collagen induction in samples incubated with dasatinib at 0.2 µmol/L versus controls (both groups each: $N = 15$ probands, $^{**}p < 0.01$).

not significantly different between controls and samples preincubated with both TKIs.

Discussion

Here we demonstrate that TKIs exert different effects on platelet function in whole blood of healthy volunteers. While imatinib significantly increased collagen-induced aggregation, nilotinib and bosutinib significantly increased collagen- and arachidonic acid–induced aggregation. Dasatinib demonstrated the most striking effect by impairing collagen-induced platelet aggregation and ATP release in a concentration-dependent manner and significantly prolonged PFA-100 closure times.

Our observation of significantly increased collagen-induced aggregation by imatinib, while other platelet activation parameters stayed unchanged, contrasts with observations in CML patients under imatinib treatment. So far, no significant changes of collagen-induced aggregation in samples from CML patients receiving imatinib were found.[19,28] Furthermore, when comparing samples of CML patients prior to and during imatinib therapy, it has been shown that imatinib does not change collagen-induced aggregation.[29] In platelet-rich plasma (PRP) from healthy volunteers, a significant decrease of collagen-induced aggregation could be shown, albeit with much higher imatinib concentrations ranging from 10 up to 160 µmol/L.[15] These imatinib concentrations by far surpass therapeutic relevant trough levels of 1 µmol/L and even peak levels of 4 µmol/L.[30,31]

Regarding reports about the effect of nilotinib and bosutinib on platelet function, no significant impact on platelet aggregation in PRP from healthy donors has been published so far.[15,24,32] Additionally, no significant effect on PRP aggregation from CML patients neither under nilotinib nor under bosutinib treatment has been found.[19,28,33] And while in vitro results do not allow a direct translation into clinical effects, there exist numerous case reports of arterial vascular occlusions for nilotinib[34–36] as well as reviews proclaiming a higher incidence of cardiovascular events under bosutinib and nilotinib therapy.[28,35,37] Additionally, whole blood aggregometry is much more suitable to detect an impairment rather than an increase of platelet function. However, flow cytometry studies performed on human (bosutinib) and murine (nilotinib) platelets showed signs of platelet activation for both TKI, but as pointed out by Deb et al. TKI effects show a high degree of interindividual variability.[11,15] Furthermore, an increase in thrombus formation could be shown for nilotinib in in vivo studies.[15] Besides platelet activation, at least for nilotinib, these higher rates of cardiovascular events might be explained by other effects on endothelial cells and lipid metabolism.[16,28,38]

Concerning dasatinib, our observations are in line with results of in vitro PRP studies demonstrating significant inhibition of platelet aggregation under dasatinib.[15,18,32] Interestingly, the inhibitory effect on aggregation in PRP already appears at much lower dasatinib concentrations from 0.05 µmol/L upward.[15] This could be due to the missing interaction of other blood cells with platelets and the

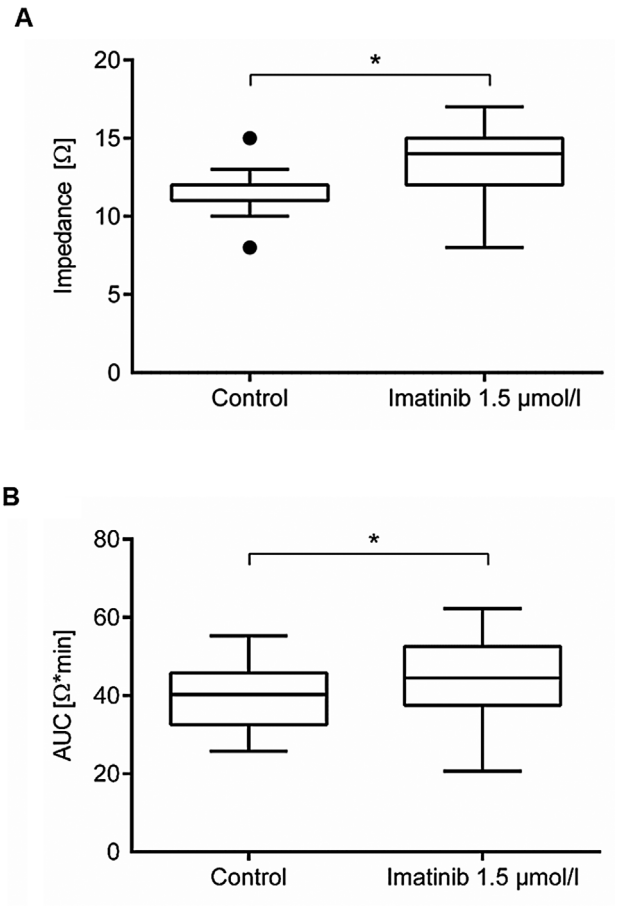

Fig. 3 (**A**) Collagen-induced maximum platelet aggregation in samples incubated with imatinib versus controls (both groups each: $N = 15$ probands, $^{*}p < 0.05$). (**B**) Area under the curve (AUC) after collagen induction in samples incubated with imatinib at 1.5 µmol/L versus controls (both groups each: $N = 15$ probands, $^{*}p < 0.05$).

absence of their amplifying effects on platelet aggregation in PRP.[39] Furthermore, a significant impairment has been shown in PRP for dasatinib concentrations from 0.15 µmol/L upward on collagen-induced ATP release.[18,32] Similar results have been obtained in blood samples from CML patients. One hour after oral dasatinib intake — a time where peak serum levels of dasatinib are present[40] — collagen-induced aggregation as well as ATP release was significantly impaired.[32] In samples taken 4 hours after oral dasatinib intake, the lumiaggregometry showed no significant differences, underlining the concentration dependency and reversibility of the effect.[32] Furthermore, an impairment of platelet aggregation was also observed in whole blood impedance aggregometry performed with the Multiplate system.[41] Additionally, we found a significant prolongation of the PFA-100 closure times for the collagen/epinephrine cartridge after incubation with dasatinib confirming prior results.[19,42] An overview of all observed TKI effects on platelet aggregation, ATP release, and PFA-100 closure times from the literature can be found in ►**Supplementary Table S1** (available in the online version).

Observed inhibitory differences of TKI might be related to their general in vitro inhibition profile. While dasatinib

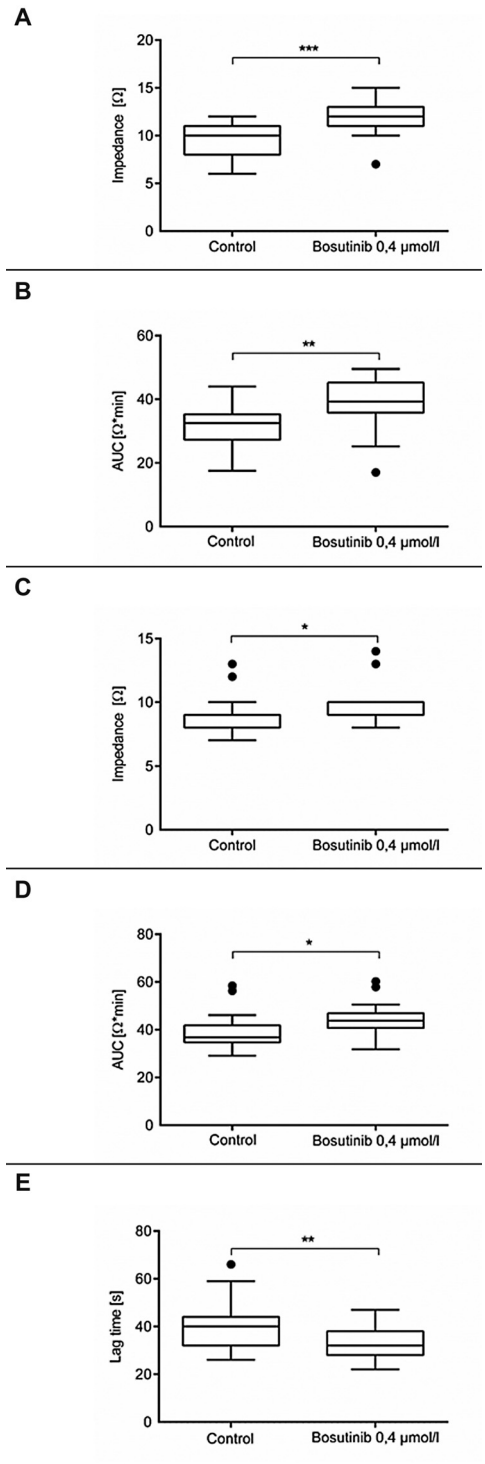

Fig. 4 (**A**) Collagen-induced maximum platelet aggregation in samples incubated with bosutinib versus controls (both groups each: $N = 15$ probands, $^{***}p < 0.001$). (**B**) Area under the curve (AUC) after collagen induction in samples incubated with bosutinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{**}p < 0.01$). (**C**) Arachidonic acid–induced maximum platelet aggregation in samples incubated with bosutinib versus controls (both groups each: $N = 15$ probands, $^{*}p < 0.05$). (**D**) AUC after arachidonic acid induction in samples incubated with bosutinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{*}p < 0.05$). (**E**) Lag time after arachidonic acid induction in samples incubated with bosutinib at 0.4 µmol/L versus controls (both groups each: $N = 15$ probands, $^{**}p < 0.01$).

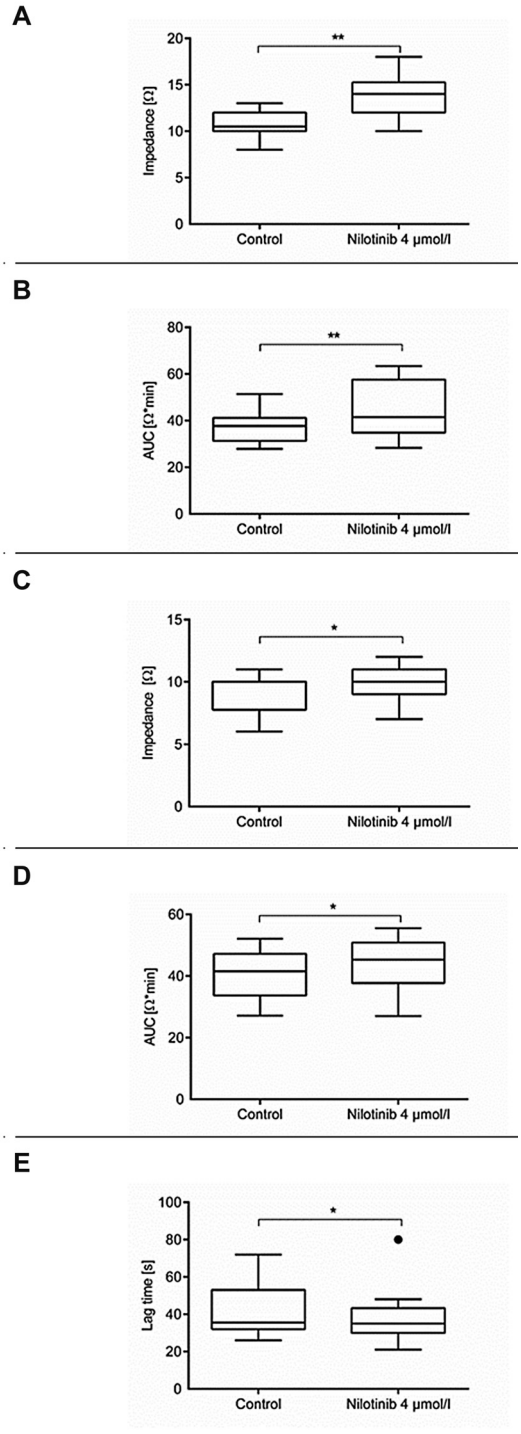

Fig. 5 (**A**) Maximum platelet aggregation after collagen induction in samples incubated with nilotinib at 4 µmol/L versus controls (both groups each: $N = 14$ probands, $^{**}p < 0.01$). (**B**) Area under the curve (AUC) after collagen induction in samples incubated with nilotinib at 4 µmol/L versus controls (both groups each: $N = 14$ probands, $^{**}p < 0.01$). (**C**) Maximum platelet aggregation after arachidonic acid induction in samples incubated with nilotinib at 4 µmol/L versus controls (both groups each: $N = 14$ probands, $^{*}p < 0.05$). (**D**) AUC after arachidonic acid induction in samples incubated with nilotinib at 4 µmol/L versus controls (both groups each: $N = 14$ probands, $^{*}p < 0.05$). (**E**) Lag time after arachidonic acid induction in samples incubated with nilotinib at 4 µmol/L versus controls (both groups each: $N = 14$ probands, $^{*}p < 0.05$).

inhibits SRC family kinases, which play a critical role in platelet activation,[18,43] imatinib and nilotinib do not exert this effect. Interestingly, bosutinib also inhibits SRC family kinases[7] but does not show inhibitory activity on platelet function.

The results from our study may give only an explanation for bleeding symptoms observed during dasatinib therapy. For the remaining TKI, we could not show an impairment of platelet function as a possible reason for clinical bleeding symptoms. As explained, the available data on the impact of imatinib, bosutinib, and nilotinib on platelet function are somewhat inconclusive.[11,16,41] Additionally, some studies suggest that the observed TKI effects on platelet function do not directly correlate with clinical bleeding symptoms.[44,45] One explanation for this missing correlation might be the high degree of interindividual variability of the observed TKI effects.[11,41] Whether an inhibitory effect can be expected with the third-generation TKI ponatinib is only speculative yet. Ponatinib was not included in our investigation, as (1) ponatinib is not licensed for first-line treatment of CML in adults or children; (2) given that the platelet counts are in the normal range, only a small minority of patients who received ponatinib experienced clinical bleeding and in none of these cases did the bleeding appear to be directly related to ponatinib[46]; (3) the action of ponatinib on platelets has been investigated extensively by other groups and ponatinib was classified to induce only a slight decrease in formation of procoagulant platelets.[11,47]

Conclusion

Here we clearly demonstrate a concentration-dependent inhibition of collagen-induced platelet activation by dasatinib in whole blood. The mechanism by which dasatinib affects platelet activation remains yet unknown, but it is suspected that its off-target inhibition on SRC family kinases plays a role. On the contrary, both nilotinib and bosutinib, which has similar off-target effects on SRC kinases as dasatinib, showed signs of platelet activation indicating a prothrombotic effect as shown previously in flow cytometry studies and clinical reviews. Concerning imatinib, while we found a significantly increased collagen-induced aggregation, in the literature both no effect and a decrease in aggregation parameters are described. But as the inhibition of platelet aggregation was shown only in supratherapeutic imatinib concentrations and most studies demonstrated no significant effect on platelet activation under imatinib, we conclude that imatinib at therapeutic relevant concentrations does not affect platelet function. The correlation of the described in vitro findings with clinical observations appears to be challenging, due to interindividual differences in TKI effects.

Ethical Vote

Institutional Ethical Board Committee, permit number EK 23012015 (date: April 28, 2015).

Authors' Contributions

The concept of this investigation was developed by all authors. F.E. performed all laboratory work as part of his medical doctoral thesis, sought informed consent, and collected blood from the voluntary donors. J.T.T. supervised the laboratory work and assisted with data interpretation. F.E. and R.K. collected and managed all data. R.K. and M.S. supervised all approaches, assisted F.E. with writing the first draft of the manuscript, and critically revised all comments. All authors revised the text and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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