Dasatinib enhances megakaryocyte differentiation but inhibits platelet formation

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Running title: Dasatinib affects platelets and megakaryocytes
Abstract

Dasatinib is an oral potent ATP-competitive inhibitor of Bcr-Abl, cKIT and Src family kinases which exhibits good efficacy in patients with Imatinib-resistant chronic myelogenous leukemia. Dasatinib treatment is associated with mild thrombocytopenia and an increased risk of bleeding but its biological effect on megakaryocytopoiesis and platelet production is unknown. In this study, we show that Dasatinib causes mild thrombocytopenia in mice without altering platelet half-life suggesting that Dasatinib inhibits platelet formation. On the other hand, the number of megakaryocytes in bone marrow of Dasatinib-treated mice is increased and the ploidy of megakaryocytes derived from bone marrow progenitor cells in vitro is elevated in the presence of Dasatinib. Furthermore, a significant delay in platelet recovery following immune-induced thrombocytopenia is observed in Dasatinib-treated mice even though the number of megakaryocytes in bone marrow is increased relative to controls at all time points. Interestingly, migration of megakaryocytes towards a gradient of SDF1α and the formation of proplatelets in vitro are abolished by Dasatinib. We propose that Dasatinib causes thrombocytopenia as a consequence of ineffective thrombopoiesis promoting megakaryocyte differentiation on one hand but impairing megakaryocyte migration and proplatelet formation on the other hand.

Keywords: Dasatinib, megakaryocytopoiesis, thrombocytopenia, proplatelet formation

Abbreviations: BM, bone marrow; CML, chronic myelogenous leukemia; MK, megakaryocyte; MLC, myosin light chain; PDGF-β, platelet-derived growth factor-β; PLCγ2, phospholipase C γ2; SCF, stem-cell factor; SDF1α, stromal cell-derived factor 1α; SFK, Src family kinases; TPO, thrombopoietin
Introduction

Dasatinib is a novel potent ATP-competitive inhibitor of multiple tyrosine kinases including Bcr-Abl, Src family kinases (SFKs) (e.g. Fyn, Yes, Src and Lyn), c-KIT, ephrin A receptor and platelet-derived growth factor-β (PDGF-β) receptor kinases. It is widely used for the treatment of Imatinib-resistant chronic myelogenous leukemia (CML). CML is a malignant proliferative disorder of hematopoietic stem cells, which is characterized by the presence of a constitutive activated form of the Abl tyrosine kinase due to a fusion product between Bcr and Abl resulting from the translocation between chromosome 9 and 22 that is the hallmark of this disease. Dasatinib is a second generation tyrosine kinases inhibitor, a more potent inhibitor of Bcr-Abl than Imatinib and with activity against other kinases, including SFKs. Side effects such as myelosuppression, gastrointestinal symptoms, diarrhea and fluid retention are commonly observed. The risk of bleeding and thrombocytopenia with Dasatinib has been clearly established among patients with CML with reported fatal brain haemorrhages and gastrointestinal bleeding. However, the biological effect of Dasatinib on megakaryocytopoiesis and platelet production to explain this observation remains uncharacterized.

Megakaryocytopoiesis is a continuous developmental process of platelet production, in which hematopoietic stem cells undergo proliferation and differentiation. Megakaryocytes (MKs) are terminally differentiated hematopoietic cells responsible for platelet production. They are formed in the proliferative osteoblastic niche of the bone marrow (BM) from hematopoietic progenitor cells. Mature MKs migrate to the vascular-rich niche where they bind to BM endothelial cells and generate proplatelets, which protrude into the bloodstream, with the final stage of platelet formation occurring in the blood. Megakaryocytopoiesis and platelet production are regulated by a variety of cytokines and chemokines. The primary cytokine regulating megakaryocytopoiesis is thrombopoietin (TPO), which binds to its
cognate receptor c-Mpl to regulate proliferation and differentiation of MK progenitors and their maturation into proplatelet-forming cells 11, 12. The chemokine, stromal cell-derived factor 1α (SDF1α), plays a vital role in the migration of MKs from the proliferative osteoblastic niche to the vascular niche through its receptor CXCR4 8, 13, 14.

Six members of the Src family of tyrosine kinases have been shown to be expressed in MKs and platelets 15, 16. SFKs play critical roles in platelet activation by a variety of glycoprotein receptors, including GPVI, CLEC-2, αIIbβ3 and GPIb-IX-V. This includes a key role in mediating changes in cytoskeletal organization leading to cell spreading and motility 17. Recently, we have demonstrated that SFKs also play a critical role in integrin-induced MK spreading, migration and activation of PLCγ2 in primary BM-derived MKs 18. MKs treated with inhibitor of SFKs are unable to spread or migrate towards a gradient of SDF1α 18. Thus, if inhibition of SFKs has the same effect in vivo, then this might account for the mild thrombocytopenia associated with Dasatinib treatment while the increase in bleeding tendency would also be explained by inhibition of platelet activation by glycoprotein receptors 19-21.

In this study, we have investigated the effect of Dasatinib on megakaryocytogenesis and platelet production in a murine model. We show that Dasatinib causes thrombocytopenia in mice to a similar level of that observed in human patients and we confirm that this is due to a defect in platelet production rather than a shortened platelet half-life. Further, we also show that MK differentiation in vitro is increased in the presence of Dasatinib but that MK migration and proplatelet formation are abolished. We therefore conclude that the thrombocytopenia observed in Dasatinib-treated patients is the result of an impairment of MK migration and proplatelet formation rather than a defect in MK growth or an increase in platelet consumption.
Materials and Methods

Chemicals

Recombinant murine stem cell factor (SCF), TPO and SDF1α were purchased from PeproTech (London, UK). Sheep anti-rat IgG Dynabeads, biotin-conjugated rat anti-mouse CD45R/B220, purified rat anti-mouse CD16/CD32, FITC-conjugated anti-mouse GPIIb, streptavidin-PE and rat anti-mouse GPIIb antibodies were from BD Pharmingen (Oxford, UK). Anti-mouse Ly-6G and biotin anti-mouse CD11b antibodies were from eBioscience (Wembley, UK). FITC-conjugated anti-mouse CXCR4 and goat anti-rat IgG FITC antibodies were obtained from R&D Systems (Abingdon, UK). Goat anti-rat IgG Alexa Fluor 488, rhodamine-phalloidin, Stempro medium and Dulbecco’s modified Eagle’s medium were from GIBCO, Invitrogen (Paisley, UK). Anti-mouse GPIbα antibody was from Emfret Analytics (Wurzburg, Germany). Anti-PLCγ2 (DN84) and anti-Syk (BR15) polyclonal antibodies were gifts from Dr. Joseph Bolen (DNAX Research Institute, Palo Alto, CA). Dasatinib (Sprycel) was purchased from LC Laboratories (Woburn, USA). Bovine serum albumin (BSA; fatty acid-free), Ribonuclease A and biotin-N-hydroxysuccinamide (Biotin-NHS) were purchased from Sigma-Aldrich (Gillingham, UK). Bovine plasma fibronectin was purchased from Calbiochem (Nottingham, UK). The anti-Src pan, anti-SFK activation loop phospho-Tyr-418 and anti-Src phospho-Tyr-529 antibodies were obtained from Invitrogen (Paisley, UK). The anti-phospho-MLC (Thr18/Ser19) and anti-MLC antibodies were purchased from Cell signalling technology (Hertfordshire, UK).

Mice

Wild type littermates were used as controls. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986 (Project License No: 40/2721, 40/9038). CD41-YFPki/+ mice were generated as
previously described\textsuperscript{10, 22}. For \textit{in vivo} imaging, the experimental procedures performed on animals were undertaken with the requirements of the German legislation.

**Platelet aggregation**

Blood was collected from CO\textsubscript{2}-asphyxiated mice by cardiac puncture into heparin (10U/ml) and PRP was prepared by centrifugation at 200 \textit{g} for 6 minutes. Platelet aggregation was measured using lumi-aggregometer (Chrono-Log, Havertown, PA, USA).

**Preparation and culture of mouse megakaryocytes**

Mature MKs from mouse BM were defined as the population of cells generated using the methodology previously described\textsuperscript{18, 23, 24}. In brief, BM cells were obtained from femora and tibiae of mice by flushing, and cells expressing one or more of the lineage specific markers on their surface (CD16/CD32\textsuperscript{+}, Gr\textsubscript{1}\textsuperscript{+}, B220\textsuperscript{+}, CD11b\textsuperscript{+}) were depleted using immunomagnetic beads (sheep anti-rat IgG Dynabeads). The remaining population was cultured in 2.6\% serum-supplemented Stempro medium with 2 mM L-glutamine, penicillin/streptomycin and 20 ng/mL murine SCF at 37\textdegree{}C under 5\% CO\textsubscript{2} for 2 days. Cells were then cultured for a further 4 days in the presence of 20 ng/mL of SCF and 50 ng/mL of TPO. After 4 days of culture in the presence of TPO, the cell population was enriched in mature MKs using a 1.5\% / 3\% BSA gradient under gravity (1\times g) for 45 minutes at room temperature as described\textsuperscript{18}.

**Megakaryocyte ploidy and flow cytometry**

Expression levels of GPIIb and CXCR4 were measured by flow cytometry using specific antibodies. Polyploidy of mature MKs isolated by BSA gradient was analyzed after anti-GPIIb labelling and DNA staining with 0.01 mg/mL propidium iodide. GPIIb positive cells
were gated to analyze DNA content. Samples were acquired using FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Oxford, UK) and analyzed using Summit v4.3 software (DAKO, Cambridge, UK).

**Cell migration assay**

Chemotaxis was assessed using the Dunn chemotaxis chamber (Weber Scientific International, Teddington, UK) as described previously 25. To investigate the effect of Dasatinib in migration, the Dunn chamber outer well was filled with the medium containing SDF1α (300 ng/mL) and the Src family kinases inhibitor Dasatinib (10 μM). Time-lapse images were digitally captured every minute for 3 hours using a Zeiss 20× 1.40 NA plan-apochromat lens on a Zeiss Axiovert 200 inverted high-end microscope (Welwyn Garden City, UK) and a Hamamatsu Orca 285 cooled digital camera. Slidebook (3I, http://www.intelligent-imaging.com/) and Image J (http://rsb.info.nih.gov/ij/) softwares were used to acquire and process images.

**Immune thrombocytopenia**

Thrombocytopenia was induced in 8-12 weeks old WT and Dasatinib-treated mice (5 mg/kg/day) by intraperitoneal injection of anti-mouse GPIbα antibody (2 μg/g of mouse), as previously described 18, 26-28. Blood samples were collected pre-injection (time = 0) and then at 3, 48, 72, 96, 120, 144 and 172 hours post-injection by tail bleeding. Platelet counts were measured using an ABX Pentra 60 Haematology Analyzer (Block Scientific, Inc).

**Platelet half life**

Measurement of platelet lifespan was performed as previously described 29, 30. Mice were injected intravenously with 150 μl of 4 mg/mL biotin-NHS. At various time points after
injection, whole blood was collected into buffer containing 10% FBS and 5 mM ethylenediaminetetraacetic acid (EDTA). 10 μl of blood was washed, pelleted at 1200 g for 10 minutes, and stained with anti-mouse GPIIb-FITC and streptavidin-PE for 1 hour on ice. Samples were washed again and the percentage of biotin-labelled platelets (GPIIb+ biotin+) was determined by flow cytometry. Platelet survival was estimated from the decay curve of the percentage of biotinylated platelets over time.

Megakaryocyte spreading and proplatelet formation

Coverslips were coated with fibronectin (20 µg/mL), fibrinogen (100 µg/mL) or BSA (100 µg/mL) overnight at 4°C, blocked with denatured BSA (5 mg/mL) for 1 hour at room temperature and washed with PBS before use. For spreading experiments, mature MKs were incubated 15 minutes with Dasatinib (10 µM) and plated on fibronectin-coated surface for 3 hours at 37°C. For proplatelet formation, mature MKs were incubated 15 minutes with Dasatinib (10 µM) and plated on fibrinogen-coated surface for 5 hours at 37°C. Adherent MKs and MKs forming proplatelets were fixed with formalin 4%, permeabilized with triton 0.1%. Actin fibres were stained with rhodamine-phalloidin and MKs forming proplatelets with anti-mouse GPIIb-FITC antibody.

Megakaryocyte biochemistry

To study tyrosine phosphorylation events in response to adhesion to fibronectin matrix, 6 well-plates Petri dishes were coated with fibronectin (20 µg/mL) or BSA (100 µg/mL) overnight at 4°C then blocked with denatured BSA (5 mg/mL) for 1 hour. After BSA gradient, mature MKs were harvested 2 hours at 37°C and then were added to fibronectin or BSA coated dishes for 3 hours. MKs were either pre-treated with DMSO (<0.1%) or with Dasatinib (10 µM) for 15 minutes at 37°C prior to plating. MKs adherent to fibronectin or in
suspension over BSA were lysed in ice-cold immunoprecipitation buffer and lysates were subjected to immunoprecipitation assays. Samples were pre-cleared for 30 minutes at 4°C with 20 μL protein G-Sepharose (50% (w/v)). Pre-cleared supernatants were incubated with 2 μL anti-PLCγ2 pAb (DN84) or 2 μL of anti-Syk Ab (BR15) and 20 μL of protein A Sepharose and samples were rotated overnight at 4°C. The Sepharose pellet was washed 3 times in lysis buffer before addition of Laemmli standard sample buffer. Pre-cleared whole cell lysates (WCLs) and immunoprecipitates were resolved by SDS-PAGE on 4% to 12% gradient gels (Invitrogen Ltd, Paisley, UK) and immunoblotted with primary antibodies and horseradish peroxidase conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

Two-photon intravital imaging of bone marrow

Preparation of mouse calvarial BM for intravital imaging was performed according to the protocol described previously 10, 31. Mice were anesthetized with 5% Vol. Isofluran (Forene®) and 0.35 l/min oxygen. During imaging, mice were maintained under anaesthesia by intraperitoneal injection (10 mL/kg body weight) of physiologic saline containing midazolam (5 mg/kg body weight, Ratiopharm, Ulm, Germany), medetomidine (0.5 mg/kg body weight, Pfizer, Berlin, Germany) and fentanyl (1.6 µg/kg body weight, CuraMED Pharma, Karlsruhe, Germany). 2-Photon in vivo imaging was performed by a TriM Scope system (LaVision BioTec, Bielefeld, Germany) based on a Ti : Sa laser (MaiTai, Spectra Physics, Darmstadt, Germany) with TriM Scope Scanhead (LaVision BioTec, Bielefeld, Germany) to capture images through a 20× water immersion objective lens (NA =0.95, Olympus, Hamburg, Germany). BM vasculature was visualized by injection of tetramethylrhodamineisothiocyanate-dextran (TRITC-dextran, 2 MDa, Invitrogen, Karlsruhe, Germany) immediately before imaging. TRITC-dextran signal was detected at a laser
wavelength of either 800 nm or 920 nm using a Semrock Bright Line Filter 593/40 (Semrock, Rochester, New York, USA). YFP signal was detected at a laser wavelength of 920 nm using 525/50nm filter (Semrock, Rochester, New York, USA). Images were acquired with InspectorPro software (LaVisionBioTec). For three-dimensional acquisition, stacks were acquired at a wavelength 920 nm with a vertical spacing of 3 µm to cover an axial depth of 30-100 µm (for YFP). Subsequently the same stacks were acquired at a wavelength of 800 nm (for TRITC). 3D volume structures were reconstructed using Volocity (Improvision, Lexington, MA, USA) at wavelength 920 nm and 800 nm. Reconstructed 3D structures were used to measure the distance between MKs and vasculature. All mice were treated with murine TPO (ImmunoTools, Friesoythe, Germany) 8 µg/kg/day for 3 days before imaging. A previous study has already shown normal physiology of thrombopoiesis after TPO treatment.  

**Immunohistochemistry**

Femora and spleens were obtained from control and Dasatinib-treated mice. Samples were fixed in buffered formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxilin and eosin (H&E) and examined by light microscopy with a 40X objective. Cryosections (5 µm) of murine femora were fixed in 4% PFA, blocked in 3% BSA-tween 0.05%, stained with anti-mouse GPIIb (1 mg/mL) and then GPIIb was detected with secondary goat anti-rat IgG Alexa Fluor 488 antibody. Three mice were used for each condition, eight to ten field of view per tissue sample, through 5 marrow sections. Fluorescence images were obtained using a Zeiss Axiovert 200 inverted high-end microscope (Welwyn Garden City, UK) microscope with a 20X objective.
Statistical analysis

Experiments were performed a minimum of 3 times and images shown are representative data from 1 experiment. Data are shown as mean ± SEM and statistical analysis was conducted using 2-tailed Student $t$ test or Anova test. $P$ values of less than 0.05 were considered statistically significant.
Results

Dasatinib induces thrombocytopenia in mice

The effect of in vivo administration of Dasatinib on the platelet count was investigated. The dose of Dasatinib (5 mg/kg/day) causes an increase in tail bleeding times at 4 and 24 h, and with full recovery by 48 h. Consistent with this, we have shown that this dose completely blocks ex vivo collagen-induced platelet aggregation for up to 6 hours (Supplementary Figure 1). Treatment with Dasatinib caused a reduction in the platelet number over the first two days which reached a plateau at approximately 70% of the original count (Figure 1A). This is similar to the decrease in count in patients with CML treated with Dasatinib. Thrombocytopenia was reversed within 4 days upon discontinuation of treatment (Figure 1B).

Dasatinib-induced thrombocytopenia is not due to an effect on platelet consumption

To determine whether an increase in platelet consumption could be responsible for the thrombocytopenia, the half-life of circulating platelets was measured following biotinylation as previously described. Dasatinib had no significant effect on platelet consumption relative to control mice (Figure 2A). In contrast, the number of new, non-biotinylated platelets was impaired by approximately 30% in line with the reduction in platelet count described above (Figure 2B). These results demonstrate that the thrombocytopenia induced by Dasatinib is mediated by defective platelet production but not by altered platelet consumption.

Dasatinib impairs platelet recovery after immune-induced thrombocytopenia

Further experiments were undertaken to investigate whether Dasatinib also plays a role in platelet formation following an acute decrease in platelet count. To investigate this, the
platelet count was reduced to less than 5% of control levels by injection of anti-GPIbα antibody. A significant delay in platelet recovery was observed in Dasatinib-treated mice following normalization of the data for the different steady-state level of platelets relative to the control group (Figure 3). Therefore, Dasatinib not only impairs platelet production at steady state but also in conditions that require an acute increase in thrombopoiesis.

**Dasatinib treatment increases the number of megakaryocytes in bone marrow**

The effect of Dasatinib treatment on the number of MKs in murine BM was investigated. Analysis of MK sections using H&E staining (Figure 4A) or by immunofluorescence using an antibody to GPIIb (Figure 4B-C) revealed a 30% increase in MKs in BM of Dasatinib-treated mice. Similarly, there was a significant increase in the number of MKs in the spleen (Figure 4A). Thus, the decrease in platelet count is not due to a reduction in the number of MKs.

The number of MKs in BM of mice treated with the anti-GPIbα antibody was also measured. In control animals, the number of MKs was reduced by over 50% within 3 hours and returned to control levels by 7 days (Figure 4B-C). The decrease in MK number in immunodepleted control mice is believed to be mediated by increased formation of both proplatelets and platelets by a pool of existing mature MKs present in the BM. In marked contrast, mice treated with Dasatinib had a similar number of MKs in BM over the same time period suggesting that the increase in formation of both proplatelets and platelets is a tyrosine kinase regulated event (Figure 4B-C).

Together, these results lead us to hypothesize that Dasatinib promotes MK differentiation but impairs the final stages of platelet production possibly by impairing MK migration and/or proplatelet formation.
The effect of Dasatinib on megakaryocytopoiesis, megakaryocyte migration and proplatelet formation in vitro

The molecular basis of the effects of Dasatinib were further investigated using primary cultures of MKs grown from BM-derived progenitors cells as previously described. Dasatinib (10 μM) was used at a concentration that is known to be sufficient to inhibit collagen-induced platelet activation through a Src kinase-dependent pathway. In the presence of Dasatinib, the percentage of GPIIb positive MKs is increase by 52% (41.0 ± 2.7 % for the control versus 61.0 ± 3.4 % for Dasatinib). In addition, a significant increase in the number of cultured MKs with a ploidy of 8-64 N is observed with a reduction in those of 2 N, probably due to the effect of the drug on the survival of the early progenitors (Figure 5A and B). Thus, there was a net increase in the overall ploidy consistent with previous reports of increased megakaryocytopoiesis in mice deficient in the Src kinase Lyn and in in vitro primary cultures of BM progenitors treated with SFKs inhibitors. The increase in MK ploidy is consistent with the increase in the number of MKs in BM of Dasatinib-treated mice.

In vivo, MKs migrate from the proliferative osteoblastic niche to the capillary-rich vascular niche where proplatelet formation takes place. We have previously modelled this movement by monitoring migration of MKs on a fibronectin matrix towards a gradient of SDF1α using a Dunn Chamber. In this assay, migration of MKs was abolished in the presence of Dasatinib (Figure 6A). Furthermore, MKs also fail to spread on fibronectin and show a diminished formation of proplatelets on fibrinogen in the presence of Dasatinib (Figure 6B and C). These results are in agreement with the recent observation that the SFKs inhibitor PP1 also blocks MK migration and spreading on fibronectin. The inhibitory action of SFKs is mediated by loss of outside-in signalling from integrin αIIbβ3 resulting in inhibition of phosphorylation of Src and Syk tyrosine kinases, phospholipase C γ2 (PLCγ2)
and myosin light chain (MLC)\textsuperscript{18}. In keeping with this mechanism, we observed a loss of phosphorylation of these proteins in the presence of Dasatinib (Figure 6Di-iii).

We also considered the possibility that Dasatinib could also affect the function or expression of the SDF1\(\alpha\) chemokine receptor, CXCR4, on MKs. Treatment with the Src kinase inhibitor had no effect on expression of CXCR4 (Supplementary Figure 2A). On the other hand, Dasatinib inhibits SDF1\(\alpha\)-induced aggregation of mouse platelets (Supplementary Figure 2B). This is likely to reflect the role of Src kinases in supporting platelet aggregation by Gi-coupled receptors as recently reported for activation of human platelets by adrenaline\textsuperscript{35}. Importantly, this study demonstrated that Dasatinib did not block inhibition of adenylyl cyclase by adrenaline thereby indicating that Src kinases synergise with other Gi-regulated pathways to mediate platelet activation. In a similar way, Src kinases may contribute to migration of MKs to SDF1\(\alpha\) and loss of this could contribute to the reduced aggregation.

We therefore concluded that inhibition of MK migration and/or proplatelet formation is the likely mechanisms through which Dasatinib causes thrombocytopenia.

**Localisation of megakaryocytes in bone marrow**

To investigate the molecular basis of the thrombocytopenia in further details, we asked whether MKs from Dasatinib-treated mice are localised to the osteoblastic niche. This was investigated using 2-photon intravital microscopy in BM in the mouse skull. MKs were identified using CD41-YFP\textsuperscript{ki/‌c} mice in which enhanced yellow fluorescent protein (YFP) was expressed as a targeted transgene from the endogenous gene locus for the \(\alpha\)IIb-integrin subunit\textsuperscript{22}. As shown in Figure 7, MKs are primarily localised to BM sinusoids with no apparent difference between control and Dasatinib-treated mice. On the other hand, the number of MKs in BM of Dasatinib-treated mice was increased by approximately 30% in the
*in vivo* skull model which is in accordance with the results in femoral BM (Figure 4). The observation that the number of MKs in the BM is increased but that their distribution is seemingly unaltered supports a model in which a defect in proplatelet formation is the primary mechanism underlying the thrombocytopenia that is seen in the presence of Dasatinib.
Discussion

The present study provides several new insights on the effects of Dasatinib on megakaryocytopoiesis and platelet formation. We demonstrate that Dasatinib causes a reversible thrombocytopenia in a murine model which is in agreement with similar clinical observations reporting its thrombocytopenic effect following chronic administration to patients. The thrombocytopenia is not caused by an accelerated clearance of platelets, but is most likely due to a defect of platelet production. Supporting this observation, we demonstrate that platelet recovery following immune thrombocytopenia is delayed in Dasatinib-treated mice. This thrombocytopenia, along with the loss of platelet activation by platelet glycoprotein receptors, including the collagen receptor GPVI, is likely to underlie the increased bleeding that is seen in patients.

We examined the effect of Dasatinib on the number of Mk within the BM of mice. Histological sections of BM from treated and untreated animals clearly show that Dasatinib induces a significant increase in the number of Mk within the BM. We further substantiated our findings in vivo using immunostaining of Dasatinib treated-mice BM and confirmed a significant increase number of Mk within the BM compared with control mice. In addition, we found that following platelet depletion, Mk were inappropriately retained in the BM of Dasatinib-treated mice whilst a significant decrease in the number of Mk was observed in the BM of control animals. We therefore postulate that Dasatinib causes thrombocytopenia not by decreasing Mk production and maturation but by impairing the mechanism by which the Mk move to the vascular niche and/or interfering with the terminal stage of platelet production through the process of proplatelet formation.

Evidence in support of this proposal was further provided by a series of in vitro studies. First, Dasatinib enhanced the differentiation of cultured Mk in vitro in agreement with previous studies showing the negative role of SFKs in MK differentiation using both the
SFKs inhibitor PP1 and Lyn-deficient mice \(^{33,34}\). Second, migration in response to SDF1\(\alpha\) in the presence of the Dasatinib was abolished. This is consistent with previous studies showing a similar effect of the SFKs inhibitor PP1, although it is possible that the positive effect of Dasatinib on MK differentiation has contributed indirectly to the reduction in migration, as fully differentiated MKs have a reduced migratory capacity \(^{10,36}\). Third, spreading and proplatelet formation were abolished by Dasatinib. The likely molecular mechanism for this effect of Dasatinib is by inhibition of phosphorylation of the active site of SFKs and loss of signalling downstream of integrin \(\alpha I I b \beta 3\). These observations are consistent with previous reports showing that MK spreading, migration and the rate of platelet recovery following immune-induced thrombocytopenia, are reduced in mice deficient in the protein tyrosine phosphatase CD148. This is believed to be due the role of CD148 in regulating global SFK activity in platelets \(^{28}\).

We sought to further establish whether it is the inhibition of migration and/or the decrease in proplatelet formation that mediates Dasatinib-induced thrombocytopenia by investigation of the spatial localisation of MKs in BM. Using an \textit{in vivo} model of skull imaging, we show that MKs are primarily localised to BM sinusoids with no apparent difference between control and Dasatinib-treated mice. The number of MKs in the BM of Dasatinib-treated mice was increased which is in consistent with the results observed in femoral BM sections. The increased number of MKs in the BM and unaltered distribution provide indirect evidence that the defect in proplatelet formation may be the primary mechanism underlying the thrombocytopenia that is seen in the presence of Dasatinib. This phenomenon is particularly striking in the immune thrombocytopenia model where a pool of “reserve” mature MKs located in the vascular niche is called upon to acutely replenish platelet numbers, presumably by means of proplatelet formation. This is reflected by the
rapid and marked decreased in the number of MKs in the control animals which is not seen in
the presence of Dasatinib.

In conclusion, using complementary approaches, we have demonstrated that
inhibition of SFKs by Dasatinib treatment strongly affects MK biology \textit{in vitro} and \textit{in vivo}. We demonstrate that \textit{in vivo} Dasatinib rapidly and reversibly induces thrombocytopenia and increases the number of mature MKs in the BM. We further show that \textit{in vitro} MK migration in response to SDF1\(\alpha\), MK spreading and proplatelet formation are abolished by Dasatinib. These effects, combined with its inhibitory effects on platelet activation \textsuperscript{20,21}, may explain the bleeding in patients treated for Imatinib-resistant CML.
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Authorship

Alexandra Mazharian: designed and performed research, collected, analyzed and interpreted data, performed statistical analysis and wrote the manuscript

Cedric Ghevaert: designed and discussed the results

Lin Zhang: performed part of the in vivo experiments and discussed the results

Steffen Massberg: contributed to design the study and discussed the results

Steve P. Watson: designed and supervised the research and wrote the manuscript

Conflict of Interest

The authors declare no competing financial interests.
References


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Figure legends

**Figure 1. Dasatinib induces reversible thrombocytopenia in mice.** (A) Whole blood platelet count from control (solid line) and Dasatinib-treated mice with 5 mg/kg/day (dashed line) were monitored every day for 7 days. Error bars represent SEM, **p<0.01, n=5 for each time points.  (B) Whole blood platelet count from control (solid line) and Dasatinib-treated mice with 5 mg/kg/day (dashed line) were monitored 2 and 4 days after interruption of the drug. Error bars represent SEM, **p<0.01, n=3 for each time points.

**Figure 2. Dasatinib treated-mice exhibit a normal platelet life span.** Control (solid line) and Dasatinib-treated mice with 5 mg/kg/day (dashed line) were intravenously injected with NHS-biotin on day 0 and (A) the percentage of biotinylated platelets was determined at indicated times by flow cytometry. (B) Relating this value to the determined platelet count yielded the number of non biotinylated platelets. Error bars represent SEM, **p<0.01, n=5 for each time points.

**Figure 3. Dasatinib treated-mice exhibit a delay in platelet recovery following immune-induced thrombocytopenia.** Whole blood platelet count from control (solid line) and Dasatinib-treated mice with 5 mg/kg/day (dashed line) mice were obtained followed immune-induced thrombocytopenia by an intraperitoneal injection of anti-mouse GPIbα antibody (2 μg/g of mouse). Platelet counts were then measured at indicated times after injection. Error bars represent SEM, n=5 for each time point. **p<0.01.
Figure 4. Dasatinib induces an increased number of mature megakaryocytes in bone marrow and spleen. (A) Representative longitudinal sections of whole murine femora and spleen stained with hematoxilin and eosin (H&E) from control mice and Dasatinib-treated mice with 5 mg/kg/day for 7 days are shown (scale bar = 50 μm). (B) Representative longitudinal sections of whole murine femora from control mice versus Dasatinib-treated mice with 5 mg/kg/day, undepleted versus platelet depleted following immune-induced thrombocytopenia by anti-GPIbα antibody (2 μg/g of mouse) at various time points are shown. MKs were identified by anti-mouse GPIIb-FITC antibody (scale bar = 20 μm). (C) Quantification of the number of MKs in the BM from control and Dasatinib treated-mice, undepleted versus platelet depleted is shown. The average number of MKs per field was determined in GPIIb stained marrow sections throughout the length of 3 femora, ***p<0.005.

Figure 5. Dasatinib increases megakaryocyte ploidy in vitro. Progenitors cells isolated from murine BM were treated with Dasatinib 10 μM along with thrombopoietin (TPO) corresponding at the beginning of the differentiation process and the inhibitor concentration was repeated 24 hours later. Four days after addition of TPO, DNA ploidy was analysed using flow cytometry by staining purified BM-derived mature MKs with propidium iodide and anti-mouse GPIIb-FITC antibody. (A) Representative profiles and (B) quantification of the percentage of cells with differing levels of ploidy and the modal ploidy from four independent experiments are shown, **p<0.01.

Figure 6. Dasatinib impairs megakaryocyte migration in response to a SDF1α gradient, spreading, proplatelet formation and integrin-induced tyrosine phosphorylation. (A) Purified BM-derived mature MKs adherent on fibronectin (20 μg/mL) coated coverslip were
allowed to migrate towards a SDF1α gradient over 3 hours within the Dunn chamber where the outer well contains SDF1α (300 ng/mL) and Dasatinib (10 μM), as described in Methods. The migration paths over 3 hours were traced. The intersection of the x- and y-axis was taken to be the starting point of each cell path, whereas the source of SDF1α was at the top. The net translocation distance (displacement from the start to the end point) of each cell in the absence or presence of Dasatinib is represented, *** p<0.005. (B) Purified BM-derived mature MKs were incubated in the presence or absence of Dasatinib (10 μM) for 15 minutes at 37°C. MKs were plated on fibronectin-coated surface for 3 hours at 37°C. Adherent MKs were fixed, permeabilized and actin fibres stained with rhodamine-phalloidin. Representative images and surface area quantification from four independent experiments are shown (scale bar = 20 μm), ** p<0.01. (C) Purified BM-derived mature MKs were incubated in the presence or absence of Dasatinib (10 μM) for 15 minutes at 37°C. MKs were plated on fibrinogen-coated surface for 5 hours at 37°C, fixed and labelled with an anti-mouse GPIIb-FITC antibody. Representative images, percent of MKs forming proplatelet and proplatelet mean area were quantified and data are presented as mean SEM of four independent experiments, ** p<0.01. (D) Purified BM-derived mature MKs were pre-incubated for 15 minutes with Dasatinib (10 μM), plated on fibronectin (FN) or BSA-coated dish for 3 hours. (i) MKs were lysed and whole cell lysates (WCL) were western blotted with SFK activation loop p-Tyr-418, Src inhibitory site p-Tyr-529 and pan Src antibodies. (ii) Syk and PLCγ2 were immunoprecipitated from equal amounts of whole cell lysates and blotted with an anti-phosphotyrosine antibody. Membranes were subsequently stripped and re-blotted with anti-Syk and anti-PLCγ2 antibodies. (iii) MKs were lysed and whole cell lysates (WCL) were western blotted with myosin light chain (MLC)-P and MLC antibodies. Western blots are representative of three independent experiments.
**Figure 7. Localisation of megakaryocytes in bone marrow of Dasatininb-treated mice.**

Localization of MKs *in vivo* is visualized by 2-photon intravital microscopy, using mouse skull BM. The MKs (green) were identified using CD41-YFP<sup>ki/+</sup> mice in which enhanced yellow fluorescent protein (YFP) was expressed as targeted transgene from the endogenous gene locus for CD41, a MK- and platelet-specific integrin. BM vasculature (red) was visualized by injection of TRITC-dextran (2 MDa) immediately before imaging. The average number of MKs per field and the percentage of MKs adjacent to the BM vasculature were determined in CD41-YFP<sup>ki/+</sup> mice control versus Dasatinib-treated mice, (scale bar = 20 μm), ***p<0.005.
Figure 1

A

Drug interruption

B

Drug interruption
Figure 2

A

- control mice
- Dasatinib-treated mice

% biotinylated platelets

0 1 2 3 4 5
days

B

Non-biotinylated platelets ($\times 10^6$/mL)

0 200 400 600 800 1000

0 1 2 3 4 5
days

** **
Figure 3

![Graph showing peripheral platelet count (x10^6/mL) over days for control mice and Dasatinib-treated mice. The graph indicates a significant difference (*) between the two groups.](image-url)
Figure 4

A

<table>
<thead>
<tr>
<th>Femoral sections (H&amp;E staining)</th>
<th>Spleen sections (H&amp;E staining)</th>
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<tbody>
<tr>
<td><strong>control mice</strong></td>
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<td><strong>Dasatinib-treated mice</strong></td>
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Figure 4

B

<table>
<thead>
<tr>
<th>Undepleted</th>
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<th>Dasatinib-treated mice</th>
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<tr>
<td>3 hours</td>
<td></td>
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<tr>
<td>1 day</td>
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<td>7 days</td>
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Figure 4

C

![Graph showing the number of megakaryocytes per field over time after platelet depletion for control mice and Dasatinib treated-mice.](image)

- **x-axis**: Time after platelet depletion (undepleted, 3 hrs, 1 day, 2 days, 7 days)
- **y-axis**: Number of megakaryocytes per field

- **Legend**:
  - Gray bars: control mice
  - Black bars: Dasatinib treated-mice

- **Significance**:
  - *** indicates statistical significance.
Figure 5

A

control

Counts

Dasatinib

B

%age

Ploidy

Count

Modal ploidy

Control

Dasatinib

**

*
Figure 6

A

control  Dasatinib

SDF1α gradient

Displacement (μm)

control  Dasatinib

B

control  Dasatinib

Phalloidin staining

Surface area (μm²)

control  Dasatinib

C

control  Dasatinib

GPIIb-FITC staining

Percent of megakaryocytes forming proplatelet

Proplatelet mean area (μm²)

control  Dasatinib
Figure 6

D

Table 1: Western Blot Analysis

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>Dasatinib</th>
<th>BSA</th>
<th>FN</th>
<th>BSA</th>
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<tbody>
<tr>
<td>i</td>
<td>WCL blot: SFK activation p-Tyr</td>
<td>blot: Src p-Tyr-529 (Inhibitory tyrosine)</td>
<td>blot: Src</td>
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<td>iii</td>
<td>WCL blot: phospho-MLC</td>
<td>blot: MLC</td>
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</tbody>
</table>
Control mice

Dasatinib-treated mice

Skull model (live imaging)

Number of megakaryocytes per field

% of megakaryocytes adjacent to the blood vessels

Figure 7

Dasatinib-treated mice

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Dasatinib enhances megakaryocyte differentiation but inhibits platelet formation

Alexandra Mazharian, Cedric Ghevaert, Lin Zhang, Steffen Massberg and Steve P. Watson