Dasatinib reversibly disrupts endothelial vascular integrity by increasing non-muscle myosin II contractility in a ROCK-dependent manner

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Abbreviations: CML, chronic myeloid leukemia; EC, endothelial cell; HUVEC, human umbilical vein endothelial cells; MLC, myosin light chain; NMII, non-muscle myosin II; ROCK, Rho-associated protein kinase; TKI, tyrosine kinase inhibitor

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Abstract

Purpose: Dasatinib is a short-acting dual ABL/SRC family tyrosine kinase inhibitor (TKI), which is frequently used to treat chronic myeloid leukemia. Although very effective, dasatinib often displays severe adverse effects, including pleural effusions and increased risk of bleeding primarily in the gastrointestinal tract. The actual causes of these side effects are currently undetermined. We hypothesize that endothelial cells (ECs) that line the inner walls of blood vessels and control the traffic to the underlying tissues, might be involved.

Experimental design: The effects of TKIs on ECs were studied by various assays, such as real-time cell impedance measurements, live-cell microscopy, wound healing, western blot and an in vivo model.

Results: Dasatinib uniquely causes a profound, dose-dependent disorganization of the EC monolayers. Dasatinib promoted the disassembly of cell-cell contacts, altered cell-matrix contacts and further altered the wound healing. A key observation is that this effect is fully reversible after drug washout. In line with these in vitro observations, intraperitoneal administration of dasatinib to mice caused significant vascular leakage in the intestine. The underlying molecular mechanism of dasatinib-induced reorganization of the actin involves ROCK activation, which increases the amount of the phosphorylation of myosin light chain and consequently activates the non-muscle myosin II.

Conclusions: Our data are consistent with a scenario in which dasatinib triggers a transient increase in vascular leakage that probably contributes to adverse effects such as bleeding diathesis and pleural effusions.
Statement of translational relevance

We describe here the cellular basis and molecular mechanism underlying dasatinib-induced pleural effusions and bleeding diathesis, which are some of the most common adverse effects of this short-acting dual ABL/SRC tyrosine kinase inhibitor (TKI). Our data demonstrate that clinically relevant concentrations of dasatinib, but not other TKIs, disrupt the endothelial monolayer and increase its permeability both in vitro and in vivo. Importantly, these effects are dose-dependent and rapidly reversible after drug washout. The ability to increase endothelial permeability transiently open new possibilities to use dasatinib in other malignancies, a clear example being co-adjuvant therapy to favor delivery of targeted treatments into solid tumors. Finally, targeted kinase inhibition has dramatically changed the treatment of haematological malignancies. Given that TKI therapy is potentially lifelong, a detailed characterization of the pathogenesis of the most frequent and serious adverse events is critical to the clinical management and to optimize the outcomes of the patients.
Introduction

Dasatinib is a short-acting tyrosine kinase inhibitor (TKI) approved for the treatment of chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia. Dasatinib is a more potent inhibitor of the oncoprotein BCR-ABL1 than other approved, first-line TKIs, such as imatinib.(1) In addition to BCR-ABL1, dasatinib inhibits a broad range of kinases, including members of the SRC, TEC, and SYK families.(2) Moreover, dasatinib has an unique half-life of only 3-4 h and the peak plasma concentration (around 100 nM) is achieved already 1-2 h after oral intake of the drug.(3, 4) Although dasatinib is very effective in the treatment of Ph+ leukemias, it displays frequent adverse effects such as diarrhea, pleural effusion, lymphocytosis, thrombocytopenia and increased risk of bleeding, which primarily involves the gastrointestinal tract.(5-7) Most of these events subside if the treatment with dasatinib is withdrawn. Importantly, the mechanisms underlying these side effects have not been elucidated, but are commonly attributed to the effect of dasatinib on off-target kinases expressed in cells other than leukemic blasts.

Endothelial cells (ECs) line the inner walls of the blood vessels, acting as an active barrier that controls blood vessel permeability. They also mediate the controlled exchange of substances between the blood and the underlying tissues, for example nutrients in the gut, and oxygen and carbon dioxide in the lungs. In addition, ECs mediate leukocyte diapedesis, enabling the ingress of leukocytes into tissues. This process guarantees the physiological homing of leukocytes and the onset of the inflammatory reaction. During inflammation, the adhesiveness of active leukocytes as well as the permeability of the endothelial monolayer increases. However, pathological sustenance of the inflammatory process as well as other factors can further maintain endothelial permeability increased, leading to edema, hemorrhage and deleterious leukocyte infiltration.(8)

The impermeability of the endothelial barrier is maintained by the strength of cell-cell junctions. Close to the lumen of the blood vessel, ECs display tight junctions mediated by occludins and claudins. At the center of the contact, cadherin-dependent adherent junctions seal the contact. ECs express VE-cadherin (CD144), which constitutes a canonical marker of EC junctions. A major regulator of junctional integrity is the actin cytoskeleton, best illustrated by the increased permeability induced by treatment of EC monolayers with actin polymerization inhibitors.(9) VE-cadherin connects with actin through alpha-catenin and the actin-binding protein vinculin.(10) These proteins are part of mechanosensitive relays that transmit myosin II-generated forces to the junctions. For example, thrombin-mediated activation of RhoA and its target Rho-associated protein kinase (ROCK) facilitates myosin light chain (MLC) phosphorylation activating non-muscle myosin II (NMII), which contracts actin at the contact, causing retraction and increasing permeability.(11) Interestingly, inhibition of contraction also increases permeability,(12) suggesting that deviations from the force balance at cadherin-
cadherin junctions compromise the barrier function. Importantly, junctions are also connected to cell-matrix adhesions through the actin cytoskeleton. ECs make discrete contacts (“focal adhesions”) with the basement membrane. (13) Focal adhesions congregate integrin receptors as well as signaling intermediates, including proteins that link integrins to the actin cytoskeleton. In the context of EC monolayers, focal adhesions participate in the barrier function by acting as anchorage points for actin cables that also connect to the VE-cadherin-dependent contacts at cell-cell junctions. (14) Thus, alterations to the cell-matrix contacts may be transmitted to the junctions, thereby perturbing permeability.

In this study, we demonstrate that dasatinib (with doses similar to those found in the plasma of patients treated with the drug) is the only TKI, which is approved for the treatment of CML, that disrupts the integrity of endothelial monolayer. This occurs through a combination of effects that include the disassembly of cell-cell contacts and the alteration of cell-matrix adhesions, that result in cells that fail to migrate collectively. (15) The disassembly of the cell-cell contacts together with the alterations to the cell-matrix adhesions are accompanied by decreased impedance of the cell monolayer, consistent with impaired integrity of the monolayer and increased permeability. Importantly, these effects are dose-dependent and reversible. Indeed, dasatinib washout restored cell-matrix adhesions, cell-cell contacts and impedance. We further show that local ROCK activation and its resulting MLC phosphorylation underlies the actin-remodeling effect of dasatinib. This unique effect of dasatinib on ECs can also be observed in vivo as mice treated intraperitoneally with dasatinib displayed vascular leakage particularly in the gut, and therefore our results bring novel insights to the pathogenesis of the adverse effects of dasatinib.
Material and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described up to the 4th passage. (16) TKIs that are currently used in the treatment of CML (dasatinib, imatinib, nilotinib, bosutinib and ponatinib)(1), were purchased from LC laboratories (Woburn, MA, USA) or Selleckchem (Houston, TX, USA). The inhibitors were used at clinically relevant concentrations and times, mimicking the half-life and the peak plasma concentration of dasatinib. (3, 4)

Measurement of endothelial cell impedance

The iCELLigence real-time cell analyzer (RTCA) measures cellular adhesion in real-time using E-plates, which are coated with high-density gold arrays for measuring electrical impedance (ACEA Biosciences Inc., San Diego, CA, USA). The iCELLigence biosensor measures cellular adhesion, which is converted to Cell Index (arbitrary units) by the iCELLigence software (version 1.1.1501). HUVECs were grown confluent on E-Plate L8-plates. Background of the E-plates was determined in 400 μl medium in the real-time cell analyzer (RTCA) station. Next, 100 μl of the HUVEC suspension was added (10,000 cells/well). E-plates were placed into the RTCA station for impedance measurement every minute for 24 h. The next day when the cells were confluent, TKIs mentioned above(1) were added to the cells at given concentrations in duplicates. E-plates were placed back into the RTCA station and measurements were done every minute for 24 h. All cell indexes were normalized (set to 1) to the last time-point before the addition of the compounds as described in the RTCA Software Manual (ACEA Biosciences Inc.)

Immunofluorescence analysis of cell-cell and cell-matrix adhesions

HUVECs were grown to confluence on gelatin-coated coverslips. Cells were then treated with the indicated dose of dasatinib, imatinib or vehicle (DMSO) for 2 h. For washouts, cells were treated for 2 h, rinsed twice with HBSS and medium replaced with inhibitor-free HUVEC medium. Cells were fixed using 4% paraformaldehyde in PBS for 10 min. For VE-cadherin staining (cell-cell contacts), antibody was diluted in TBS + 1% BSA. For actin and vinculin, cells were permeabilized for 10 min using 0.2% Triton X-100 in TBS and stained with Alexa647-phalloidin (Invitrogen, Camarillo, CA, USA) for F-actin or a 1:500 dilution of hVin-1 mAb (Sigma-Aldrich, St. Louis, MO, USA) + Alexa568-conjugated secondary antibody (Invitrogen). Cells were examined using a 4-laser (405, 488, 568, 647 nm) Leica SP5 spectral confocal scanning microscope fitted with a HyD detector (Leica Biosystems, Nussloch,
Germany) and a 63x, 1.40NA PLANAPo oil objective. Images were acquired using LAS AF software (Leica) and analyzed using ImageJ (NIH).

**Live microscopy**

HUVECs were grown to confluence on 0.2% gelatin-pretreated cover slips for 24 h and DIC images were obtained in an inverted microscope (Olympus IX83) fitted with a full-housing Pekon incubator for temperature and CO₂ control. Samples were imaged using a 20x NA 0.75 immersion (oil) objective and a Hamamatsu EM-CCD (512x512) camera. Images were sequentially collected every 60sec using Xcellence software (Olympus). Dasatinib or imatinib were added *in situ* as indicated in the figure.

**Wound healing**

HUVECs were grown to confluence in μ-Slide 4well (Ibidi) previously coated with fibronectin (3 µg/ml). Wounds were made by dragging a 10 µl sterile pipette tip across the monolayer. Cells were incubated with dasatinib (100 nM), nilotinib (5 µM), imatinib (10 µM), bosutinib (500 nM) and without TKI for 12 h. Pictures were taken every 15 min using the Olympus IX83 microscope as described in the previous section. Images were combined using a stitching algorithm (17) and analyzed using ImageJ (NIH).

**Quantification of phosphorylated myosin regulatory light chain (RLC)**

Phosphorylated RLC (Ser19) was quantified by densitometric analysis of confocal images. Briefly, condition-matched images were Z-projected using ImageJ, then analyzed for integrated intensity along a 1024x1024 image. Then, the total cellular surface was calculated by thresholding the cell contours and calculating the overall surface covered by the cell monolayer. On average, % coverage was >95% for DMSO, dasatinib+Y-27632; 85% for Y-27632; and 60-75% for dasatinib. The integrated intensity was corrected for the % coverage in each condition and values were referred to 100%, which was the mean of the measurements of cells treated with DMSO. Data represents the measurements of >10 fields (40x) from two independent experiments, corresponding to >200 cells. Statistical significance was determined according to Mann-Whitney test after every condition failed the Shapiro-Wilk normality test.

**Western blot**

Confluent HUVECs were treated with DMSO or inhibitors for the times indicated in the figure. Washouts were carried out by rinsing the cells with HBSS at 37°C, followed by incubation with complete medium for the indicated times. After incubation, cells were scraped in Laemmli 2x buffer. Lysates were separated in 7% and 13,5% SDS/PAGE gels. Samples were transferred to PVDF membranes, blocked with 4% BSA and incubated with antibodies against pThr18/pSer19...
MLC, pSer19 MLC, pThr96 MYPT1, pThr853 MYPT1 and total MYPT1 were from Cell Signaling. Antibody against total MHCII-B was from Biolegend and antibody against tubulin was from Sigma. After incubation with species-matched, HRP-conjugated antibodies and thorough rinsing with TBS-Tween, blotted images were acquired in an ImageQuant LAS-4000 workstation (GE). Bands were subjected to densitometry using ImageJ and normalized using the loading control (total MHCII-B or tubulin).

Viability assay

Confluent HUVECs were treated with dasatinib 5nM, 10nM, 25nM, 50nM and 100nM for 3h. The entire supernatant was collected and 7AAD was added. Events were acquired for 60sec from each tube with FACSVerse (BD Biosciences, San Jose, CA, USA). Simultaneously the number of detached cells was counted.

In vivo endothelial permeability assay

Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME and Envigo, Frederick, MD) were treated 3 days with dasatinib (50 mg/kg), imatinib (100 mg/kg), bosutinib (50 mg/kg) (all LC laboratories) or vehicle (Citric acid, pH 3.0) intraperitoneal once a day. At the third day, 30 min after the drug administration, Evans Blue (5 mg/ml in PBS, Sigma-Aldrich) was injected intravenously to the tail vein. The mice were euthanized after 30 min. Spleen, lungs, brains, and small intestine were collected and weighted. 0.5 ml of formamide (Sigma-Aldrich) was added and incubated at 55°C for 24 h. The intensity of Evans Blue in the organs was determined by detecting absorbance at wavelength 610 nm. The animal experiments were approved by the Animal Experiment Board of the State Provincial Office of Southern Finland. All studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Statistical analysis

The results were compared by 1-way ANOVA Kruskal-Wallis test. Significance was set at p<0.05 and all analysis were performed using GraphPad Prism software (version 5.0c; GraphPad. La Jolla, CA, USA).
**Results**

*Dasatinib reversibly disrupts the integrity of the endothelial monolayer in a dose-dependent manner*

To assess the hypothesis that the adverse effects of dasatinib are at least partially based on its effect on ECs, we studied the effects of dasatinib and other TKIs that are currently used in the treatment of CML, on the integrity of monolayers of HUVEC. This was done by impedance measurements, which is a good readout of the integrity of a monolayer. (18)

We selected different concentrations of dasatinib, imatinib, nilotinib, bosutinib and ponatinib and vehicle alone (DMSO) that mimic the actual concentrations found in the serum of TKI-treated patients. (19-21) Real-time follow-up of the cell contacts showed that the addition of dasatinib quickly decreases cell impedance in a dose-dependent manner. After 2 h of treatment with 5 nM and 10 nM dasatinib, we observed a mild decrease in impedance (Fig.1A). In contrast, 25 nM and 50 nM dasatinib induced a steep decrease, whereas 100 nM (a dose similar to the levels found in the plasma of patients 1 h after oral uptake of 100 mg dasatinib) caused a profound decrease in impedance (Fig.1A). Conversely, treatment with 2 µM imatinib for 2 h had no significant effect, and 10 µM decreased the impedance only slightly (Fig.1B). Clinically relevant concentrations of nilotinib (1 and 5 µM), bosutinib (100 and 500 nM) and ponatinib (20 and 100 nM) had no effect either on the impedance readings (Fig.1C). The inhibitors were dissolved in DMSO and diluted at least 1:5000 in cell culture media to avoid any effects to the impedance (Fig.1D).

To address whether the effect of dasatinib was due to non-reversible damage to the endothelial monolayer, we removed the inhibitor, added fresh medium and measured the impedance for another hour in the absence of dasatinib. Importantly, impedance quickly recovered to levels comparable to those measured at the beginning of the experiment (Fig.1A).

*Dasatinib induces a fast and reversible disorganization of the actin cytoskeleton and the appearance of large gaps in endothelial monolayers*

To address the possible cellular effects of dasatinib that decrease the impedance of the HUVEC monolayers, we examined the appearance of the HUVEC monolayers after treatment with the inhibitor. Initially, we stained polymerized F-actin with fluorescently-tagged phalloidin (Fig.2A). We found that 10-25 nM dasatinib induced very small gaps between cells (Fig.2A) and a modest decrease of the junctional F-actin (the F-actin found at the cell-cell contacts), consistent with the mild effect of these doses on impedance. On the other hand, 50 nM dasatinib had a much more dramatic effect. We observed a deep decrease of junctional actin and the appearance of thin, F-actin rich lamellipodia facing larger gaps between cells. Finally, 100 nM dasatinib promoted a complete loss of the integrity of the monolayer. Cells appeared as single...
entities or in small islets with a high percentage of protrusive cells as revealed by thin rims of F-actin at round edges (Fig.2A, arrowheads). Conversely, 10 µM of imatinib had no effects on the monolayer.

Since the major effect of dasatinib focused on the cell-cell junctions, we examined these in more detail by visualizing the adherens junction marker VE-cadherin, which was no longer detected on the surface of dasatinib-treated HUVECs (Fig.2B). However, dasatinib washout rapidly restored VE-cadherin at the reformed cell-cell junctions. These data indicate that dasatinib induces the rapid disappearance of VE-cadherin from the cell-cell contacts concomitant to their dissolution.

We next analyzed the dynamics of contact dissolution and reformation using time-lapse live-cell microscopy. By imaging confluent HUVECs every minute after the addition of dasatinib, we observed that the drug quickly disrupted the cell-cell contacts, and large gaps between the cells could be observed as early as 6 min after dasatinib addition (Fig.3A, upper panels). After 1 h, the endothelial monolayer was disorganized and only scattered cell-cell contacts remained. Individual, spindly, isolated cells were readily observed (Fig.3A, Supplemental video S1). Conversely, imatinib had no significant effect on the integrity of the monolayers (Fig.3A, lower panels). Again, dasatinib washout led to the rapid recovery of the integrity of the monolayer, which was apparent after 10 min and comparable to untreated monolayers after 40 min (Fig.3B, Supplemental video S2).

Dasatinib prevents endothelial cell wound healing by inhibiting the formation of cell-cell contacts

The effect of dasatinib on the integrity of the EC monolayer suggested a crucial role for a dasatinib target in the organization of polarized actin at cell-cell junctions. This led us to hypothesize that dasatinib would also prevent the formation of cell-cell contacts if the integrity of the monolayer had been compromised by alternative means, e.g. mechanical wounding of the monolayer. In this scenario, cells are required to migrate first, and then form stable contacts.

To test this hypothesis, we performed wound-healing assays in the presence of different TKIs. The wound area decreased similarly in the presence of nilotinib, bosutinib and imatinib as in the control wounds and healed completely in 420 min. Conversely, dasatinib significantly slowed down the healing process (Fig.4A-B), with ~30% of the wound still open after 12 h from scratching. Time-lapse movies of the closing wounds (Supplemental videos S3 and S4) revealed that dasatinib compromised the integrity of the advancing endothelial monolayer, promoting the emergence of isolated cells that migrated individually, i.e. detached and lacking any contact with other cells of the monolayer nearby. Careful examination of the advancing dasatinib-treated monolayers revealed loss of cell-cell contacts and mesenchymal traits in many individual...
cells, e.g. lamellipodia in cells in the rear cohorts (Fig.2A, arrowheads). These data suggest that dasatinib does not prevent cell migration, but it inhibits the formation of intercellular contacts, promoting a more individual, non-collective type of migration and thereby preventing the reestablishment of the integrity of the monolayer.

Dasatinib increases ROCK/non-muscle myosin II activity

The effect of dasatinib in the organization of the actin cytoskeleton and the disappearance of junctional actin suggested an alteration in a signaling pathway involved in actin remodeling. A major regulator of actin remodeling is NMII.(22) To test the involvement of NMII in dasatinib-induced actin remodeling, we treated HUVEC monolayers with dasatinib and other TKIs that are currently used in the treatment of CML and measured its effect on Ser19 and Thr18/Ser19 phosphorylation of MLC. These phosphorylations are bona fide markers of the activation of NMII(23), which is the main actin-related molecular motor expressed in these cells.(24) Western blot and image intensity quantification revealed that dasatinib induced a modest, but reproducible, increase in the amount of MLC phosphorylated in Ser19 and Thr18/Ser19, consistent with a dasatinib-induced reorganization of the actin by modulation of NMII activity (Fig.5A-C). These changes also altered the distribution of cell-matrix adhesions, which were sharper and better defined in dasatinib-treated cells compared to control conditions (Fig.5D). Together, these data indicate that dasatinib increases the mesenchymal traits of endothelial cells, perturbing the cell-cell junctions by increasing NMII-mediated contractility.

Next, we aimed at elucidating the specific mechanism by which dasatinib promotes these changes in endothelial monolayers. Based on the alterations in NMII phosphorylation, we hypothesized that ROCK was involved in the mechanism altered by dasatinib. To address this, we treated HUVEC cells with the specific ROCK inhibitor Y-27632 in the absence, or presence, of dasatinib. We found that, consistent with previous results, Y-27632 reversibly abrogated phosphorylation of MYPT1 on Thr696 and Thr853, which are two dependent sites through which ROCK prevent NMII dephosphorylation and inactivation (Fig.5E). Dasatinib restored some MLC phosphorylation despite the presence of Y-27632 (Fig.5A-C) and also MYPT1 phosphorylation on Thr853, and specially Thr696 (Fig.5E). These results indicate that dasatinib promotes the activation of ROCK, even overcoming the inhibiting effect of Y-27632. Also, these experiments constitute the proof of a molecular mechanism by which dasatinib promotes local ROCK activation and alters the organization of the actin cytoskeleton.

Therapeutic concentrations of dasatinib are not toxic for endothelial cells

The dramatic effects observed in the presence of dasatinib could be due to acute toxicity of the drug. A major argument against this possibility is the reversibility of the cellular effects when the drug was washed out. If dasatinib was toxic for the cells, the cells would not be able to...
recover their ability to reform cell-matrix adhesions or the integrity of the endothelial monolayer. To further rule out toxicity-related effects, we treated HUVEC with different clinically relevant concentrations of dasatinib and cell viability from 4 different ECs monolayers was assayed using 7AAD. The mean percentage of non-viable 7AAD positive cells in the control wells was 5.4%, and with 5 nM dasatinib 5.4%, 10 nM 7.5%, 25 nM 6.9%, 50 nM 7.5% and 100 nM 7.5% (p=0.026). Moreover, the number of detached cells was counted from the supernatant of each well by constant flow for 60 sec and compared to the control well (no dasatinib). No significant increase was observed in the number of cells in the supernatant in respect to the control condition (mean fold change of detached cells in respect to the control well (1) was 1.0 with 5 nM dasatinib, 1.1 with 10 nM, 1.1 with 25 nM, 1.1 with 50 nM and 1.2 with 100 nM dasatinib; p=0.13). These results are consistent with low toxicity at this dose of dasatinib on ECs.

Dasatinib causes vascular leakage in vivo

To correlate our observations in an in vivo setting, we performed an Evans Blue assay, which measures blood vessel permeability in vivo. Mice were treated intraperitoneally for 3 days with dasatinib (50 mg/kg), imatinib (100 mg/kg), or bosutinib (50 mg/kg) once a day. This dose of dasatinib causes similar side-effects, such as lymphocytosis, than those seen in humans at a therapeutic dose. On the third day, 30 min after the administration of the drugs, Evans Blue was injected intravenously. The mice were euthanized after 30 min, and different organs were collected into solvent and Evans Blue concentration was measured.

Dasatinib significantly increased the amount of Evans Blue in the small intestine compared to the control group and the other TKIs (dasatinib mean 13.6, vehicle 1.0, imatinib 2.8, bosutinib 1.5 mg Evans Blue/mg tissue; p=0.0089) (Fig.6A, supplementary Fig.S5). The amount of detected Evans Blue in the spleen was also higher in the mice which had been treated with dasatinib (dasatinib mean 11.1, vehicle 6.6, imatinib 3.9, bosutinib 7.6 mg Evans Blue/mg tissue; p=0.04) (Fig.6B). In contrast, no significant differences of Evans Blue were found in the brain (dasatinib mean 6.7, vehicle 0.3, imatinib 0.6, bosutinib 2.2 mg Evans Blue/mg tissue) and lungs (dasatinib mean 25.1, vehicle 38.9, imatinib 2.4, bosutinib 2.2 mg Evans Blue/mg tissue).
Discussion

Many studies have reported the immune-related adverse effects of dasatinib.\(^{(7, 27-33)}\) However, these side-effects do probably not account for all the observed deleterious effects of the treatment in human patients, e.g. pleural effusions and bleeding diathesis. These are critical events that physicians take into account when prescribing the drug, but data on their molecular basis remains scarce. Interestingly, we found that dasatinib, in contrast to the other studied TKIs, compromises the barrier function the vascular endothelium, which is consistent with some of the observed unique side-effects of dasatinib.

Pleural effusions are the most characteristic adverse events in dasatinib-treated Ph+ leukemia patients. Depending on the study, 10-35% of the treated patients develop pleural effusions at some point of their treatment. These effusions have traditionally been considered as unique side effect to dasatinib treatment as they are extremely rare during imatinib, nilotinib and ponatinib treatment.\(^{(34-37)}\) Recent studies showed that a small percentage of bosutinib-treated patients has pleural effusions, however, most of them had a history of pleural effusions with previous treatments with dasatinib.\(^{(38)}\) In contrast, pleural effusions are rarer in patients treated first-line with bosutinib.\(^{(39)}\) Pleural effusions may depend on different cellular systems and an increase in the endothelial barrier permeability of both vascular and lymphatic vessels in intimate contact with the visceralis pleura could contribute to the onset of pleural effusions. Interestingly, we did not observe significant differences in vascular leakage in the lungs of mice treated with different TKIs. As seen in our results, also the control mice had an elevated amount of Evans Blue in the lungs, which might be caused by contamination of blood from large vessels while removing the lungs, rendering possible differences in vascular leakage undetectable. Another possibility is that the barrier function of the lung is intrinsically less efficient than in other organs. Lung microvasculature is non-contractile and proteins of the connexin family permit passage to molecules under 1 kDa in size.\(^{(40)}\) The molecular weight of Evans Blue is close to 1 kDa, hence it is not unsurprising that it appears in the lungs of control animals. Evans Blue might therefore be inadequate to assess lung permeability, but it is excellent to account for endothelial permeability in contractile endothelium, e.g. in the gut. Moreover, the effusions observed in the patients are likely related to the cumulative, long-term use of dasatinib, as effusions normally occur after weeks of treatment and are dose-dependent.\(^{(41)}\) We propose that dasatinib causes accumulative damage and leakage due to its reversible effect on the barrier function of ECs when the clinical concentration is achieved.

Another typical adverse effect of dasatinib is the onset of hemorrhage and bleeding diathesis.\(^{(41)}\) Such events have rarely been reported in CML patients treated with other TKIs.\(^{(38, 39, 42, 43)}\) The pathophysiology of bleeding diathesis associated with dasatinib therapy remains poorly understood. Typically, this has been attributed to thrombocytopenia and/or the reversible inhibition of platelet aggregation caused by dasatinib.\(^{(44, 45)}\) Bleeding has
been observed in up to 23% of dasatinib-treated patients who had failed imatinib treatment. The
majority of the bleeding was localized to the gastrointestinal tract. Moreover, an overview of
> 900 patients undergoing treatment with dasatinib displayed hemorrhages in various tissues in
40% of the patients. Also, 14% of the patients displayed gastrointestinal bleeding. Most of these
patients were receiving high doses of dasatinib (>100mg) or two doses per day. The specific
occurrence of gastrointestinal bleedings correlates with the oral administration of dasatinib,
which therefore involves direct contact of the drug with the local gastrointestinal endothelium.
Our mice experiment reproduced these observations of the dasatinib-treated patients.

Another possible effect of the disruption of the endothelium by dasatinib in vivo is an
exposure of the subendothelial matrix, which may cause platelet immobilization and a
subsequent decrease of the number of circulating platelets. Interestingly, the platelet count in
peripheral blood drops quickly after dasatinib intake, recovering back to baseline a few hours
later. In this scenario, dasatinib-induced thrombocytopenia might be caused not only by a
central and chronic effect on megakaryocytopoiesis and platelet production, but also by an
acute peripheral, transient effect at the endothelial level.

Many different phosphatases and kinases act as regulators of the endothelial barrier function,
like those involved in the activation of NMII, including ROCK and MLC. In migrating cells,
SRC phosphorylates ROCK on Y722, decreasing its association to RhoA, thereby preventing
NMII phosphorylation via this axis. This mechanism is involved in adhesion turnover at the
leading edge. We propose a model in which a Src family kinase (e.g. SRC) phosphorylates
and inactivates ROCK on Y722, maintaining NMII activation in check to maintain the
homeostasis of cell-cell junctions. Dasatinib would inhibit this SFK, promoting ROCK
activation, which would in turn phosphorylate MYPT1 on Thr696 and Thr853. This
phosphorylation inhibits the phosphatase, increasing NMII phosphorylation. Interestingly,
dasatinib reverses the inhibitory effect of Y-27632. This could be due to an allosteric effect by
which dephosphorylation of ROCK in Y722 (caused by dasatinib) would decrease the affinity
of Y-27632 for ROCK. Alternatively, an additional kinase could be mediating the
phosphorylation of MYPT1 on Thr696 and Thr853. The latter is unlikely since ROCK
phosphorylates those residues directly.

Despite all this, these findings do not rule out additional mechanisms or other kinases.
Several possibilities emerge: one is that there is simultaneous inhibition of two, or more,
kinases, that account for a joint phenotype as described here. This can occur in an additive,
synergistic or cascading manner. Another non-exclusive possibility is that dasatinib has more
than one mechanism of inhibition of a given kinase: clear examples would be the inhibition of
scaffolding functions or the inhibition of the kinase domain of the putative kinase. In this
scenario, the blockade of the kinase activity would prevent the release of an autoinhibitory
domain (in the kinase itself, or in another adaptor), which would result in the blockade of an entire pathway.

The ability of dasatinib to open transiently and dose-dependently endothelial cell-cell junctions would not only explain some of the side-effects of the drug such as bleeding, thrombocytopenia and pleural effusions, but it would also promote its use to deliver other therapeutic drugs to previously unreachable anatomic locations. In this view, dasatinib could be useful as co-adjuvant therapy to transiently increase the endothelial barrier permeability and favor the delivery of additional targeted treatments into organs, for example solid tumors. In addition, our wound healing experiments suggest that this drug has angiostatic activity as interferes with the establishment of the endothelial monolayer and would hinder the formation of new vessels and the consequent delivery of nutrients and oxygen to the tumor.

In summary, we demonstrate that clinically relevant concentrations of dasatinib disrupt the endothelial monolayer dose-dependently and reversibly, which is accompanied with impaired integrity and increased permeability. The effect was observed also in vivo, as mice treated with dasatinib displayed vascular leakage especially in the gut. Our results bring novel insights to the pathogenesis of the typical side effects observed in dasatinib-treated patients.
Author’s Contributions:

Concept and design: A. Kreutzman, M. Vicente-Manzanares, C. Muñoz-Calleja

Development of methodology: A. Kreutzman, C. Cuesta-Mateos, M. Vicente-Manzanares, C. Muñoz-Calleja


Analysis and interpretation of data: A. Kreutzman, M. Vicente-Manzanares, C. Muñoz-Calleja

Writing, review, and revision of the manuscript: A. Kreutzman, J.L. Steegmann, S. Mustjoki, M. Vicente-Manzanares, C. Muñoz-Calleja

Administrative, technical, or material support: C. Delgado Arévalo, Y. Pérez-García, FIMM Technology Center High Throughput Biomedicine unit

Study supervision: A. Kreutzman, M. Vicente-Manzanares, C. Muñoz-Calleja

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References


Figure legends

Figure 1. Dasatinib uniquely lowers HUVEC impedance in a reversible and dose-dependent manner. HUVECs were cultured on iCELLigence E8 plates overnight to gain full confluence. When confluent, different concentrations of (A) dasatinib, (B) imatinib, (C) nilotinib, bosutinib, ponatinib or (D) DMSO were added to the wells for 2 h. Dasatinib was washed out from the wells and the cells were left to recover for another hour while recording the impedance. The cell impedance was measured every minute with iCELLigence for 24 h. The experiment shown is representative of three performed.

Figure 2. Dasatinib disorganizes actin cytoskeleton and breaks cell-cell junctions in a dose-dependent and reversible manner. HUVECs were cultured on cover slips pre-coated with gelatin until confluent. (A) Dasatinib dose-response was analyzed by visualizing the actin-cytoskeleton by microscope. When confluent, the cells were treated with dasatinib for 2 h at different concentrations (or imatinib 2 h, 10 uM). The wells were then fixed and further stained with Alexa647-phalloidin to detect F-actin. (B) Confluent HUVECs were treated with 100 nM dasatinib or 10 uM imatinib for 2 h. After the treatment, some wells were washed three times with warm medium and the cells were recovered in cell culture conditions for 1 h. The wells were then fixed and further stained for VE-Cadherin. F-actin was simultaneously stained to observe the concomitant disorganization of the actin cytoskeleton. All images were taken with a Leica DMR fluorescence microscope fitted with a Xenon lamp and a 20x immersion (oil) objective. Images are representative of 20 fields acquired per condition in three independent experiments. Scale bar, 50 µm

Figure 3. Live imaging on the effect of dasatinib on cell-cell junctions. HUVECs were cultured on cover slips pre-coated with gelatin. When confluent, the cells were placed in a microscope, and 100 nM dasatinib or 10 uM imatinib were added. Images were taken every minute for 1 h. After 1 h, the cells were washed three times and recovered while obtaining images every minute for 1 h. The live images were obtained in an inverted microscope (Olympus IX83) fitted with a full-housing Pekon incubator to control temperature and CO₂. Samples were imaged using a 20x NA 0.75 immersion (oil) objective and a Hamamatsu EM-CCD (512x512) camera.

Figure 4. Dasatinib impairs wound closure and alters collective cell migration. (A) HUVECs were grown to confluence in μ-Slide 4-well and scratched with a 2 μl-pipet tip in the presence of the doses of dasatinib, imatinib, nilotinib and bosutinib as indicated in Material and Methods. Percentage of open area either for dasatinib, imatinib, bosutinib and control every
hour is represented. Data is the mean ± SD of two independent experiments in which we represent the % area of the coverslip not covered with cells at every time point. (B) Representative images of the experiments quantified in (A), control and dasatinib wound at 0, 240, 450 and 720 min.

Figure 5. Cell-cell junction dissolution induced by dasatinib involves non-muscle myosin II activation and altered ROCK activity. (A) HUVECs were grown to confluence in tissue culture dishes, then treated with the indicated doses of the indicated inhibitors for 1 h. In the wash conditions, cells were incubated with the inhibitors for 1 h, removed and allowed to recover for an additional 1 h. Cells were lysed and blotted for MLC phosphorylated on Ser19 or Thr18/Ser19 as indicated. Tubulin is shown as loading control. The experiment shown is representative of three performed. (B) Confocal images of HUVECs grown to confluence in gelatin-covered coverslips and incubated with the indicated inhibitors (100 nM dasatinib, 20 uM Y-27632, alone or in combination) for 1 h. Cells were fixed and stained for MLC phosphorylated on Ser19 and F-actin. Scale bar 50 µm. (C) Quantification by densitometric analysis of fields as shown in (B) (See material and methods for details). Data represents the measurements of >10 fields (40x) from two independent experiments, corresponding to >200 cells. Statistical significance was determined according to Mann-Whitney test. (D) Confocal images of HUVECs grown to confluence in gelatin-covered coverslips and incubated with DMSO or 100 nM dasatinib for 2 h. Cells were fixed and stained for vinculin to visualize focal adhesions. Images are representative of 20 fields acquired per condition in three independent experiments. Scale bar, 50 µm. (E) HUVECs grown to confluence in tissue culture dishes were treated with Y-27632 (20 uM), alone or in combination with dasatinib for 1 h. In the wash condition, inhibitors were removed and cells allowed to recover for an additional 1 h. Cells were lysed and blotted for phosphorylated MYPT1 (residues Thr696 and Thr853). MHCII-B (myosin II heavy chain) is shown as a loading control. The experiment shown is representative of three performed.

Figure 6. Dasatinib causes endothelial leakage in vivo. Blood vessel permeability was studied by an Evans Blue assay. The mice were treated ip. 3 days either with dasatinib (50 mg/kg), imatinib (100 mg/kg), bosutinib (50 mg/kg) or vehicle, and albumin binding Evans Blue was injected iv. After 30 min, the mice were sacrificed, and spleen, lungs, brains, and small intestine were collected into solvent and Evans Blue concentration was measured. Increased concentration of Evans Blue was observed in the (A) small intestine and (B) spleen of dasatinib-treated mice.
Figure 1

A

B

C

D

Normalized Cell Index

Normalized Cell Index

Normalized Cell Index

Normalized Cell Index

+dasatinib

dasatinib

washout

+imatinib

+imatinib

+TKI

+DTKI

no dasatinib

dasatinib 5nM

dasatinib 10nM

dasatinib 25nM

dasatinib 50nM

dasatinib 100nM

no imatinib

dasatinib

dasatinib 2uM

dasatinib 10uM

no imatinib

dasatinib

Imatinib 2uM

Imatinib 10uM

media

DMSO

Nilo 1uM

Nilo 5uM

Bosu 100nM

Bosu 500nM

Pona 20nM

Pona 100nM

media

DMSO

1:100000

1:50000

1:10000

1:5000

1:1000

1:500

1:100
**Figure 3**

**A**

Dasatinib 100nM

- Pre-treatment
- 1min
- 6min
- 11min
- 31min
- 51min

Imatinib 10uM

- Pre-treatment
- 1min
- 6min
- 11min
- 31min
- 51min

Inhibitor added

**B**

Dasatinib washout

- 0min recovery
- 10min recovery
- 20min recovery
- 40min recovery
Figure 5

A

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C

![Graph showing Ser19 phosphorylation](image)

- p<0.01
- p=0.06
- p=0.01
Figure 6

A | Small intestine
---|---
Vehicle | Dasatinib | Imatinib | Bosutinib
mg Evans Blue/Mg tissue

B | Spleen
---|---
Vehicle | Dasatinib | Imatinib | Bosutinib
mg Evans Blue/Mg tissue

*p* = 0.0089

*p* = 0.0439
Dasatinib reversibly disrupts endothelial vascular integrity by increasing non-muscle myosin II contractility in a ROCK-dependent manner


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