Dasatinib Reversibly Disrupts Endothelial Vascular Integrity by Increasing Non-Muscle Myosin II Contractility in a ROCK-Dependent Manner

Anna Kreutzman1,2, Beatriz Colom-Fernández1, Ana Marcos Jiménez1, Mette Ilander2, Carlos Cuesta-Mateos1, Yaiza Pérez-García1, Cristina Delgado Arévalo1,3, Oscar Brück2, Henna Hakanen4, Jani Saarela4, Alvaro Ortega-Carrion1,3, Ana de Rosendo1, Alba Juanes-García1,5, Juan Luis Steegmann5, Satu Mustjoki2, Miguel Vicente-Manzano6, and Cecilia Muñoz-Calleja1

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, patients taking dasatinib often display 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 vitro 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. Clin Cancer Res; 23(21):6697–707. ©2017 AACR.

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 a unique half-life of only 3 to 4 hours, and the peak plasma concentration (around 100 nmol/L) is achieved already 1 to 2 hours after oral intake of the drug (3, 4). Although dasatinib is very effective in the treatment of Ph+ leukemias, patients display 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10). These proteins are part of VE-cadherin connects 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—actin-binding protein vinculin (10).
Dasatinib Disrupts Adhesive Structures on Endothelial Cells

For actin and vinculin, cells were permeabilized for 10 minutes using 0.2% Triton X-100 in TBS and stained with Alexa647-phallolidin (Invitrogen) for F-actin or a 1:500 dilution of hVin-1 mAb (Sigma-Aldrich) + 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) and a 63×, 1.40NA PLANAPO 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 hours, 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 20× NA 0.75 immersion (oil) objective and a Hamamatsu EM-CCD (512 × 512) camera. Images were sequentially collected every 60 seconds using Xcellence software (Olympus). Dasatinib or imatinib was added in situ as indicated in Figure 3.

Wound healing
HUVECs were grown to confluence in μ-Slide 4-well (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 nmol/L), nilotinib (5 μmol/L), imatinib (10 μmol/L), bosutinib (500 nmol/L), and without TKI for 12 hours. Pictures were taken every 15 minutes using the Olympus IX83 microscope as described in the previous section. Images were combined using a stitching algorithm (17) and analyzed using Imager (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 1,024 × 1,024 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, 90% of the coverage was measured for DMSO, dacatinib + 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 represent the measurements of 10 fields (40 ×) from two independent experiments, corresponding to >200 cells. Statistical significance was determined according to the 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 Figure 5. 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 2× 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 pThr853 MYPT1, pSer19 MLC, pThr18/pSer19 MLC, pThr696 MYPT1, and total MYPT1 were from Cell Signaling Technology. 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 5, 10, 25, 50, and 100 nmol/L for 3 hours. The entire supernatant was collected, and 7AAD was added. Events were acquired for 60 seconds from each tube with FACSVerse (BD Biosciences). Simultaneously, the number of detached cells was counted.

In vivo endothelial permeability assay
Female C57BL/6 mice (The Jackson Laboratory and Envigo) 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 minutes 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 minutes. 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 hours. 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 one-way ANOVA Kruskal–Wallis test. Significance was set at P < 0.05, and all analyses were performed using GraphPad Prism software (version 5.0c; GraphPad).

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 hours of treatment with 5 and 10 nmol/L dasatinib, we observed a mild decrease in impedance (Fig. 1A). In contrast, 25 and 50 nmol/L dasatinib induced a steep decrease, whereas 100 nmol/L (a dose similar to the levels found in the plasma of patients 1 hour after oral uptake of 100 mg dasatinib) caused a profound decrease in impedance (Fig. 1A). Conversely, treatment with 2 μmol/L imatinib for 2 hours had no significant effect, and 10 μmol/L...
decreased the impedance only slightly (Fig. 1B). Clinically relevant concentrations of nilotinib (1 and 5 μmol/L), bosutinib (100 and 500 nmol/L), and ponatinib (20 and 100 nmol/L) had no effect either on the impedance readings (Fig. 1C). The inhibitors were dissolved in DMSO and diluted at least 1:5,000 in cell culture media to avoid any effects on 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 with 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 to 25 nmol/L 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 nmol/L 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 nmol/L 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 μmol/L of imatinib had no effects on the monolayer.

Because 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 minutes after dasatinib addition (Fig. 3A, top). After 1 hour, the endothelial monolayer was disorganized and only scattered cell–cell contacts remained. Individual, spindly, isolated cells were readily observed (Fig. 3A; Supplementary video S1). Conversely, imatinib had no significant effect on the integrity of the monolayers (Fig. 3A, bottom). Again, dasatinib washout led to the rapid recovery of the integrity of the monolayer, which was apparent after 10 minutes and comparable with untreated monolayers after 40 minutes (Fig. 3B, Supplementary 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 minutes. Conversely, dasatinib significantly slowed down the healing process (Fig. 4A and B), with ~30% of the wound still open after 12 hours from scratching. Time-lapse movies of the closing wounds (Supplementary 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 with 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 four different EC monolayers was assayed using 7AAD. The mean percentage of non-viable 7AAD-positive cells in the control wells was 5.4%, and with 5 nmol/L dasatinib 5.4%, 10 nmol/L 7.5%, 25 nmol/L 6.9%, 50 nmol/L 7.5%, and 100 nmol/L 7.5% ($P = 0.026$). Moreover, the number of detached cells was counted from the supernatant of each well by constant flow for 60 seconds and compared with 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 nmol/L dasatinib, 1.1 with 10 nmol/L, 1.1 with 25 nmol/L, 1.1 with 50 nmol/L and 1.2 with 100 nmol/L 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 (25). 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 (26). On the third day, 30 minutes after the administration of the drugs, Evans Blue was injected intravenously.
The mice were euthanized after 30 minutes, 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 with 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, for example, 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 patients with dasatinib-treated Ph+ leukemia. Depending on the study, 10% to 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 visceral peritoneum 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

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 hour. In the wash conditions, cells were incubated with the inhibitors for 1 hour, removed and allowed to recover for an additional 1 hour. 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 nmol/L dasatinib, 20 μmol/L Y-27632, alone or in combination) for 1 hour. 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 Materials and Methods for details). Data represent the measurements of >10 fields (40 ×) 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 nmol/L dasatinib for 2 hours. 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 μmol/L), alone or in combination with dasatinib for 1 hour. In the wash condition, inhibitors were removed and cells allowed to recover for an additional 1 hour. Cells were lysed and blotted for phosphorylated MYPT1 (residues Thr696 and Thr853). MHCI-B (myosin II heavy chain) is shown as a loading control. The experiment shown is representative of three experiments performed.
Dasatinib causes endothelial leakage in vivo. Blood vessel permeability was studied by an Evans Blue assay. The mice were treated i.p. 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 i.v. After 30 minutes, 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.

Dasatinib disrupts adhesive structures on endothelial cells. Figure 6. Blood vessel permeability was studied by an Evans Blue assay. The mice were treated i.p. 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 i.v. After 30 minutes, 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 6. Dasatinib causes endothelial leakage in vivo. Blood vessel permeability was studied by an Evans Blue assay. The mice were treated i.p. 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 i.v. After 30 minutes, 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.

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 anatomical locations. In this view, dasatinib could be useful as a coadjuvant 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 it 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 endothelial barrier function, like those involved in the activation of NMII, including ROCK and MLIC. 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 (48). We propose a model in which an Src family kinase (e.g., SRC) phosphorylates and inactivates ROCK on Y722, thus preventing NMII activation (49). 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 because ROCK phosphorylates those residues directly (49).

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 nonexclusive 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.
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.

Disclosure of Potential Conflicts of Interest
H.I. Steegmann is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, and Pfizer. S. Mustjoki reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, and Pfizer, and reports receiving commercial research grants from Ariad, Novartis and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: A. Kreutzman, C. Cuesta-Mateos, S. Mustjoki, M. Vicente-Manzanares, C. Muñoz-Calleja
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Colom-Fernández, A. Marcos Jiménez, M. Ilander, Y. Pérez-García, C. Delgado Arévalo, O. Brück, H. Hakanen, J. Saarelä, A. Ortegacarrion, A. de Rosendo, J. A. J. Saareselä, A. Juanes-Garcia, S. Mustjoki, M. Vicente-Manzanares

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

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Marcos Jiménez, C. Delgado Arévalo
Study supervision: A. Kreutzman, S. Mustjoki, C. Muñoz-Calleja

Acknowledgments
The authors would like to thank Dr. Paloma Sánchez-Mateos for providing HUVECs, the personal at the Department of Immunology, Hematology Research Unit Helsinki and the FIMM Technology Center High Throughput Biomedicine unit for their technical expertise, support, and use of instruments.

Grant Support
A. Kreutzman is a Sigrid Jusélius postdoctoral fellow. Grants PI12/00494P and PI15/02085 from the Fondo de Investigaciones Sanitarias to C. Muñoz-Calleja supported this work. C. Muñoz-Calleja was co-financed by FEDER funds. M. Vicente-Manzanares is an investigator from the Ramon y Cajal Program (RYC-2010-06094) and is supported by grants SAJ/2014/4705-R from MINECO, Marie Curie CIG-293719 from the EU, Q1616A1831 from Ramon Areces Foundation and the BBVA Foundation. S. Mustjoki is supported by the Finnish Cancer Institute, Academy of Finland, Sigrid Jusélius Foundation and Finnish Cancer Associations.

The costs of publication of this article were deferred in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 15, 2016; revised August 10, 2016; accepted August 10, 2017; published OnlineFirst August 18, 2017.
Dasatinib Disrupts Adhesive Structures on Endothelial Cells


24. Kolega J Asymmetric distribution of myosin IIb in migrating endothelial cells is regulated by a rho-dependent kinase and contributes to tail retraction. Mol Biol Cell 2003;14:4745–57.


Dasatinib Reversibly Disrupts Endothelial Vascular Integrity by Increasing Non-Muscle Myosin II Contractility in a ROCK-Dependent Manner


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-0667

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2017/08/18/1078-0432.CCR-16-0667.DC1

Cited articles
This article cites 47 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/23/21/6697.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/23/21/6697.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/23/21/6697. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.