Dasatinib inhibits pro-inflammatory functions of mature human neutrophils

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Running title: Dasatinib inhibits neutrophil functions

Scientific category: Phagocytes, Granulocytes, and Myelopoiesis

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ABSTRACT

Dasatinib is a tyrosine kinase inhibitor used to treat imatinib-resistant chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. At present, little is known about how dasatinib influences non-malignant cells. Here we tested the effect of dasatinib on functional responses of normal mature human neutrophils. Dasatinib completely blocked integrin- and Fc-receptor-mediated neutrophil functions with the lowest IC$_{50}$ values below 10 nM under serum-free conditions. Dasatinib caused a partial inhibition of neutrophil responses triggered by G-protein-coupled receptors and had a moderate effect on neutrophil responses triggered by microbial compounds. While dasatinib inhibited neutrophil chemotaxis under static conditions in two dimensions, it did not affect migration under flow conditions or in three-dimensional environments. Dasatinib did not have any major effect on phagocytosis or killing of bacteria by neutrophils. Adhesion of human neutrophils in the presence of whole serum was significantly inhibited by 50-100 nM dasatinib which corresponds to reported serum concentrations in dasatinib-treated patients. Finally, ex vivo adhesion of mouse peripheral blood neutrophils was strongly reduced following oral administration of 5 mg/kg dasatinib. Those results suggest that dasatinib treatment may affect pro-inflammatory functions of mature neutrophils and raise the possibility that dasatinib-related compounds may provide clinical benefit in neutrophil-mediated inflammatory diseases.
INTRODUCTION

Dasatinib (BMS-354825) is a second-generation oral tyrosine kinase inhibitor primarily used as a second-line treatment in imatinib-resistant chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia\(^1\). Similar to imatinib, dasatinib also inhibits the Abl kinase and the Bcr-Abl fusion protein though with a different molecular mechanism of action\(^2,3\). In addition to Abl and Bcr-Abl, dasatinib also inhibits a number of additional kinases including Src- and Btk-family members, c-Kit, the PDGF-receptor and Eph receptors\(^3,4\).

In addition to its effect on malignant cells, dasatinib also inhibits certain functions of normal cells of various hematopoietic lineages including T-lymphocytes\(^5\), NK-cells\(^6\), basophils\(^7\), platelets\(^8,9\) and osteoclasts\(^10\). However, no information is available on the effect of dasatinib on neutrophils, the most abundant circulating leukocytes.

Neutrophils are short-lived, terminally differentiated phagocytic cells that provide the first line of defense against bacterial and fungal pathogens but also contribute to the development of various acute and chronic inflammatory diseases\(^11,12\). Neutrophil activation occurs through a number of cell surface receptors (integrins, Fc-receptors, G-protein-coupled, cytokine- and innate immune receptors) which activate complex intracellular signal transduction events leading to cellular responses such as adhesion, migration, respiratory burst, granule release, phagocytosis and bacterial killing.

A number of genetic and pharmacological studies indicate that protein tyrosine kinases play critical roles in neutrophil activation by various cell surface receptors\(^13\). Src-family kinases are involved in neutrophil functions triggered through integrins\(^14-16\) or formyl-peptide receptors\(^15,17\). Pharmacological studies suggested a role for Abl in integrin-mediated activation\(^18\), L-selectin shedding\(^19\) and respiratory burst\(^20\) of neutrophils. We and others have identified critical roles for Syk in various neutrophil activation pathways\(^16,21-24\).

The lack of information on the effect of dasatinib on neutrophils the putative role of Src-family kinases and c-Abl in neutrophil activation, and the inhibition of certain neutrophil functions by dasatinib in a kinase inhibitor screening study (K. F., T. V., György Kéri and A. M., unpublished observations) prompted us to perform a detailed analysis of the effect of dasatinib on human neutrophil functions. Our results indicate that dasatinib exerts a robust inhibitory effect on various inflammation-related functions of mature human neutrophils.
MATERIALS AND METHODS

Neutrophil isolation and inhibitor treatment

Human neutrophils were isolated from venous blood of healthy volunteers by Ficoll or Percoll gradient centrifugation followed by hypotonic lysis of red blood cells17,25. Cells were resuspended in Ca²⁺- and Mg²⁺-free HBSS supplemented with 20 mM HEPES, pH 7.4 and kept at room temperature until use.

Dasatinib (>99% pure) was obtained from Selleck Chemicals and its purity and stability was confirmed by HPLC-MS analysis (Vichem Ltd., Budapest, Hungary). Dasatinib was dissolved in DMSO. The final dasatinib-treated samples contained ≤0.01% DMSO whereas inhibitor-free controls contained 0.01% DMSO.

Isolated neutrophils were diluted in the assay medium, supplemented with 0.5 mM CaCl₂, and then pretreated with the indicated concentrations of dasatinib or vehicle (DMSO) at 37 °C for 30 min prior to activation. Unless otherwise stated, 1 mM MgCl₂ was added immediately before cell activation. Neutrophil assays were performed at 37 °C with dasatinib being present throughout the assays. Cell viability and the basal rate of apoptosis for up to 6 hours was not affected by up to 1 µM dasatinib (data not shown).

All experiments on human samples were approved by the Institutional Review Board of the Semmelweis University or the Ludwig-Maximilians University.

Neutrophil activation

Plate-based activation of neutrophils was performed in Nunc Maxisorp or tissue culture-treated BD Biosciences plates. For adherent activation of neutrophils, the plates were pre-coated with 150 µg/ml human fibrinogen (Calbiochem) or 10% FCS (Invitrogen), and stimulated with 20 ng/ml human TNF-α (TNF; Peprotech), 50 ng/ml human C5a (R&D Systems), 1 µg/ml Pam3CSK₄ (EMC Microcollections), 1 µg/ml ultrapure LPS (InVivoGen) or 100 nM PMA (Sigma) as described 15,21,26,27. Neutrophil activation by plate-bound anti-integrin antibodies (20 µg/ml anti-CD18 clone IB4) or the polyvalent integrin ligand poly-RGD (20 µg/ml; Sigma) was performed as described21,26,28. Immobilized IgG immune complexes were prepared using human lactoferrin (Lfr; 20 µg/ml) and anti-Lfr (1:400 dilution) or, in the case of measuring lactoferrin release, human serum albumin (HSA; 20 µg/ml) and anti-HSA (1:400 dilution; all reagents from Sigma) and used to stimulate neutrophils as described29.

Neutrophil activation in suspension was performed in polypropylene tubes or in FCS-coated wells in the absence of MgCl₂ by stimulating the cells with 20 ng/ml TNF, 1 µM fMLP (Sigma), 50 ng/ml C5a, 100 ng/ml human IL-8 (Peprotech), 50 ng/ml LTB₄ (Santa Cruz), 1 µg/ml Pam₃CSK₄, 1 µg/ml ultrapure LPS or 10 mg/ml zymosan (Sigma)
opsonized or not with normal or heat-inactivated human serum. Where indicated, cells were pretreated with 10 µM cytochalasin B (Sigma).

**Functional assays and biochemical studies**

For respiratory burst assays, cells were supplemented with 100 nM ferricytochrome c (Sigma), plated at 10^5/well on 96-well plates and their superoxide release was monitored spectrophotometrically as described^21,22,26,27^. Alternatively, the samples were supplemented with 50 µg/ml lucigenin (Sigma) and their luminescence was followed using a Thermo Labsystems Fluoroskan Ascent FL luminometer. Cell spreading was assessed after 30 min stimulation by phase contrast microscopy of formalin-fixed cells using a Leica DMI 6000B inverted microscope. Cellular adhesion during a 30 min incubation was determined after several washes using an acid phosphatase assay as described^21^. Lactoferrin release was tested using a double-sandwich ELISA^17^ whereas gelatinase release was followed by in-gel gelatinase zymography^16,29^ after 10 (fMLP) or 30 min (all other stimuli) activation. Upregulation and activation of CD11b during a 30 min incubation was assessed using biotinylated anti-human CD11b (M1/70) followed by streptavidin-FITC (both from BD Biosciences) or FITC-labeled antibodies against an activation-specific human CD11b epitope (clone CRBM1/5; eBioscience). Samples were analyzed using a BD Biosciences FACSCalibur flow cytometer and the CellQuest software.

Phosphorylation of intracellular proteins was tested on cell lysates prepared using a Triton X-100-based lysis buffer supplemented with protease and phosphatase inhibitors^26^ after incubation of the cells for 3 min (fMLP), 5 min (IL-8, C5a, LTB₄), 10 min (TNF, Pam₃CSK₄, zymosan) or 20 min (upLPS) in suspension or for 10 min (immune complex) or 15 min (TNF on fibrinogen and poly-RGD) on a solid surface. Where indicated, Syk was immunoprecipitated using the 4D10 monoclonal antibody (Santa Cruz) and Protein A/G-Sepharose beads (Invitrogen) as described^21,26^. Total cell lysates or Syk immunoprecipitates were immunoblotted using anti-phosphotyrosine antibody (clone 4G10; Millipore), phospho-specific antibodies against Syk (#2701; Cell Signaling), or phospho-specific (Cell Signaling) or non-phospho-specific (Santa Cruz) antibodies against the ERK and p38 MAP-kinases as described^21,22,26^.

**Neutrophil migration**

The migration of individual neutrophils towards a gradient of 10 µM fMLP or 1 µg/ml IL-8 during a 10-min period at 37 °C on immobilized fibrinogen (250 µg/ml) under steady-state conditions was tested using a Zigmond chamber assay as described^27,30^.
Mechanotactic crawling of human neutrophils was analyzed using IBIDI µ-slides VI 0.4 flow chambers coated with 250 µg/ml human fibrinogen or 12.5 µg/ml human ICAM1 (Peprotech). Cells were treated with 1 µM fMLP for 10 min inside the chamber before application of 1 dyne/cm² shear stress for 10 min using a high-precision syringe pump (KD Scientific). Time-lapse video microscopic images recorded using a Zeiss Axiovert 200 microscope were analyzed off-line using the NIH ImageJ software with manual tracking plugin (Fabrice Cordeliès, Institute Curie, Orsay, France). Single cell migration tracks were analyzed using IBIDI chemotaxis and migration tools.

Transwell migration assays were performed essentially as described using polycarbonate filters with 3 µm pore size (Corning) precoated with human fibrinogen. Neutrophil migration towards 100 nM fMLP or 10 ng/ml IL-8 in 60 min was quantified using an acid phosphatase assay. To assess migration through an extracellular matrix, neutrophils were allowed to migrate through Transwell inserts filled with 100 µl 8-fold diluted Matrigel (BD Biosciences) for 3 hours.

Bacterial killing and phagocytosis

Killing of S. aureus or E. coli opsonized with pooled human serum and incubated with neutrophils for 30 min at a neutrophil:bacteria ratio of 1:10 was tested by a plate-based assay as described. For phagocytosis assays, GFP-expressing S. aureus bacteria were opsonized and incubated with neutrophils as above for 0, 10 or 20 min, washed and resuspended in BD FACS Lysis buffer, followed by determination of neutrophil-associated fluorescence by flow cytometry. Where indicated, neutrophils were preincubated with 10 µM cytochalasin D (Sigma).

Adhesion of unfractionated human and mouse leukocytes

To test adhesion of unmanipulated leukocytes in the presence of whole serum, red blood cells were sedimented from heparinized human blood by 0.4% Dextran 500 (Sigma), the leukocyte-rich supernatant was treated with dasatinib and incubated on an FCS-coated surface in the presence of 20 ng/ml human TNF, 100 ng/ml human C5a, 1 µg/ml Pam3CSK4 or 1 µg/ml ultrapurified LPS. After 30 min at 37 °C, the plates were washed and leukocyte adhesion quantified using an acid phosphatase assay.

To test the effect of oral administration of dasatinib, adult C57BL/6 mice were treated with the indicated doses of dasatinib by oral gavage. Two hours later, the mice were treated with heparin, sacrificed, and leukocyte-rich plasma was obtained by Dextran sedimentation of peripheral blood as described above. Ex vivo adhesion of leukocytes to FCS-coated plates in the presence of 50 ng/ml murine TNF was tested as described above.
Animal experiments were authorized by the Semmelweis University Animal Experimentation Review Board.

Presentation of data and statistical analysis

All experiments were performed three or more times with comparable results. Respiratory burst assays were performed in triplicates. Zero time points and unstimulated control values were subtracted to simplify presentation of kinetic curves. Other quantitative assays were performed in duplicates or triplicates. In case of representative graphs, error bars represent SD from a single experiment. Densitometry was performed using the ImageJ software.

For generation of percent response values and dose-response curves, unstimulated control values were subtracted, the responses expressed in percent of vehicle-treated samples, and the data averaged across the indicated number of experiments with error bars representing SEM. In case of kinetic experiments, dose-response curves were generated using the last time point or, in the case of luminometric respiratory burst assay, the integrated area under the curve. Maximum inhibition and IC\textsubscript{50} values were calculated using a four-parameter logistic algorithm by IDBS XLFit with the inhibition in the absence of dasatinib set to 0% and maximal inhibition limited to 100%.

Where indicated, statistical analysis was performed using Student's paired two-population t-test. p values below 0.05 were considered statistically significant.

RESULTS

Dasatinib blocks adhesion-mediated respiratory burst

Neutrophil activation at the site of inflammation occurs through various pro-inflammatory agonists while the cells are adherent to the inflamed endothelium or the extracellular matrix. This can be mimicked by stimulating neutrophils by soluble agonists in the presence of an adhesive surface\textsuperscript{33} (so-called adherent activation) which requires β\textsubscript{2}-integrins\textsuperscript{21,26,34}. Proinflammatory agonists including TNF, C5a, and TLR2 (Pam\textsubscript{3}CSK\textsubscript{4}) or TLR4 (ultrapure LPS) ligands potently triggered superoxide release from human neutrophils adherent to a fibrinogen- or FCS-coated surface (Figs 1A-B). Low nanomolar concentrations of dasatinib exerted a robust inhibition of those responses with IC\textsubscript{50} values below 10 nM in the most sensitive assay conditions tested (Figs 1A-B).

Neutrophils can also be potently activated upon co-ligation of integrins and Fc-receptors by immobilized anti-integrin antibodies\textsuperscript{38,35}. As shown in Fig 1C, that response was also completely blocked by dasatinib.
In addition to the above co-stimulatory approaches, neutrophils can also be activated by plating them on a surface coated with a polyvalent integrin ligand (poly-RGD) in the absence of another proinflammatory stimulus. As shown in Fig 1D, such activation was also dramatically inhibited by dasatinib.

The above findings raise the possibility that dasatinib blocks the assembly of the NADPH oxidase or otherwise interferes with the superoxide release assay. However, respiratory burst triggered by the non-physiological activating agent PMA was not affected by dasatinib (Fig 1E) and another, luminometric respiratory burst assay confirmed complete inhibition of the TNF-induced respiratory burst of fibrinogen-adherent neutrophils (Fig 1F).

**Dasatinib inhibits other adhesion-mediated neutrophil functions**

Adherent activation of neutrophils also triggers other functional responses including spreading and adhesion of the cells and exocytosis of secondary granules. Dasatinib potently inhibited the spreading (Fig 2A-B) and adhesion (Fig 2C) response and the release of the secondary granule marker lactoferrin (Fig 2D) upon stimulation of fibrinogen-adherent neutrophils with TNF, with IC₅₀ values in the range of 35-50 nM depending on the assay readout used. In contrast, dasatinib did not affect the spreading of PMA-stimulated human neutrophils (not shown), indicating that the final cytoskeletal spreading machinery remained intact upon dasatinib treatment.

**Dasatinib blocks activation of the Syk tyrosine kinase**

Adherent activation of neutrophils triggers various tyrosine phosphorylation pathways. Tyrosine phosphorylation of cellular proteins in response to TNF stimulation of fibrinogen-adherent neutrophils (Fig 2E) or upon plating the cells on poly-RGD-coated surfaces (Fig 2F) was inhibited by 10 nM and completely blocked by 100 nM dasatinib. The only exception was an approx. 40 kDa protein in Fig 2E, likely corresponding to p38 MAP-kinase activated by TNF in an adhesion-independent manner (see below).

We have previously shown a critical role for Syk in adherent activation of neutrophils. Both TNF-mediated activation of fibrinogen-adherent neutrophils (Fig 2E) and plating the cells on a poly-RGD-coated surface (Fig 2F) triggered phosphorylation of Tyr352 of Syk and that phosphorylation was strongly reduced by as little as 10 nM dasatinib. Complete inhibition of adhesion-induced Syk phosphorylation by 100 nM dasatinib could also be observed upon immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies (Fig 2G). Given the essential role of Syk in...
adherent activation of neutrophils21, those results indicate that the effect of dasatinib is likely mediated, at least in part, by blocking the activation of the Syk tyrosine kinase.

Dasatinib does not block TNF signal transduction

The above results indicated that dasatinib blocks adhesion-induced signal transduction. To test whether it also interferes with TNF-receptor signaling, neutrophils were stimulated by TNF under non-adherent conditions. As shown in Fig 2H, dasatinib did not affect the TNF-induced exocytosis of gelatinase granules (which, in contrast to lactoferrin release, does not require cellular adhesion). Dasatinib did not substantially affect TNF-induced upregulation of CD11b at the indicated 30 min time point either (Fig 2I). Dasatinib significantly reduced the TNF-induced expression of an activation-specific CD11b neoepitope during a 30-min incubation period at 1 µM but not at 10-100 nM concentrations of the drug (Fig 2J). As shown in Fig 2K, dasatinib did not significantly affect TNF-induced phosphorylation of the ERK or the p38 MAP-kinases except for a partial inhibition of the phosphorylation of p38 MAP-kinase at the highest concentration (1 µM) tested. Those results suggest that mid-nanomolar concentrations of dasatinib do not block TNF-receptor signal transduction.

Taken together, low to mid nanomolar dasatinib concentrations inhibit various adhesion-dependent functional responses of human neutrophils (Figs 1, 2A-D) but do not significantly affect TNF signal transduction. The most likely explanation is that dasatinib inhibits integrin signal transduction, at least in part by preventing the activation of the Syk tyrosine kinase.

Dasatinib blocks immune complex-induced neutrophil activation

Immune complex formation is a major cause of neutrophil activation in autoimmune diseases12. As shown in Fig 3, dasatinib abrogated immune complex-induced spreading (Fig 3A-B), superoxide production (Fig 3C) and exocytosis of specific and gelatinase granules (Figs 3D-E) with IC50 values in the range of 25-50 nM. Dasatinib also inhibited basal and immune complex-induced tyrosine phosphorylation and activation of the p38 MAP-kinase (Fig 3F). Similar to integrin-mediated activation, Syk is also thought to be required for immune complex-induced neutrophil activation36,37. Immune complex-induced phosphorylation of the Tyr352 residue of Syk was inhibited by 10 nM and completely blocked by 100 nM dasatinib (Fig 3F) and Syk phosphorylation determined by immunoprecipitation and phosphotyrosine immunoblotting was also completely blocked by 100 nM dasatinib (Fig 3G). Taken together, mid-nanomolar concentrations of dasatinib abrogate immune complex-
induced neutrophil activation, likely at least in part by blocking the activation of the Syk tyrosine kinase.

**Partial inhibition of G-protein-coupled receptor signaling**

We next tested the effect of dasatinib on signaling by G-protein-coupled receptors that sense bacterial invasion, activation of the inflammation machinery and tissue damage (e.g. extracellular release of mitochondrial formyl peptides).

As shown in Fig 4A, dasatinib caused a considerable but not complete inhibition of superoxide production triggered by the formyl-peptide fMLP, with IC_{50} values below 10 nM both in the presence and absence of cytochalasin B (a cytoskeleton disrupting agent that augments certain neutrophil functions). Dasatinib also partially inhibited lactoferrin release in the absence but not in the presence of cytochalasin B (Fig 4B) while it did not significantly affect upregulation (Fig 4C) or activation (Fig 4D) of CD11b at the indicated time point, exocytosis of gelatinase granules (Fig 4E) or phosphorylation of the ERK or p38 MAP-kinases (Fig 4E).

We next tested neutrophil responses triggered by IL-8, C5a and LTB4, G-protein-coupled receptor agonists that do not induce robust respiratory burst. Dasatinib did not affect the upregulation (Fig 4F) or activation (Fig 4G) of CD11b triggered by IL-8. Lower concentrations of dasatinib did not affect while the highest concentration slightly reduced gelatinase release triggered by IL-8, C5a and LTB4 (Figs 4H-J). Dasatinib also partially inhibited the phosphorylation of the p38 MAP-kinase in response to IL-8, C5a and LTB4, though that inhibition was primarily seen only at the highest concentration tested (Figs 4H-J). Interestingly, while 10-100 nM dasatinib did not affect ERK phosphorylation triggered by those agonists, the 1 µM dose tended to slightly augment that response (Figs 4H-J).

Taken together, while low nanomolar concentrations of dasatinib exert a partial inhibition of certain fMLP-induced responses, other G-protein-coupled receptor-mediated neutrophils functions are either not affected or only affected at very high concentrations of dasatinib.

**The effect of dasatinib on neutrophil migration**

Neutrophils are able to accumulate at the site of inflammation by highly organized chemotactic migration. To test the effect of dasatinib on that process, we first performed video microscopic assessment of neutrophil chemotaxis in a CD18-dependent Zigmond chamber assay under static conditions. As shown in Fig 5A, migration of human neutrophils towards fMLP on a fibrinogen-coated substrate was strongly inhibited by dasatinib with IC_{50} values in the range of 15-30 nM. The migration
of neutrophils towards IL-8 under similar conditions was also strongly inhibited by 100 nM dasatinib (Fig 5B).

We next tested whether dasatinib also affected two-dimensional mechanotactic migration of neutrophils on integrin ligand surfaces under flow conditions in the presence of a homogenous concentration of fMLP. Because of the use of integrin ligands in the absence of selectin ligands, this assay models the process of intraluminal crawling whereas no leukocyte rolling was possible under the conditions used \(^{24,38}\). As shown in Fig 5C, initiation of flow caused about 50% of the adherent cells to detach from both fibrinogen and ICAM1 even in the absence of dasatinib. As expected, 100 nM dasatinib reduced both the pre-flow adhesion of neutrophils and, particularly in case of fibrinogen, also the remaining adhesion at the end of the 10-min flow period. Interestingly, the migration of cells that remained adherent through the entire 10-min flow period was not affected by 100 nM dasatinib (Fig 5D), suggesting that dasatinib inhibits neutrophil adhesion but not mechanotactic migration.

We also tested neutrophil migration through FCS-coated polycarbonate membranes in β\(_2\) integrin-dependent \(^{21}\) Transwell assays. As shown in Fig 5E, dasatinib did not inhibit the migration of neutrophils towards fMLP or IL-8 under those conditions.

The apparent contradiction between the results presented in Figs 5A-B and Figs 5D-E prompted us to test neutrophil migration in a more physiological environment, i.e. through a complex extracellular matrix (Matrigel) preparation. Placing Matrigel in Transwell inserts significantly reduced and delayed the migration of neutrophils compared to Matrigel-free inserts (compare Figs 5E-F) indicating that Matrigel did indeed form a migration barrier. Importantly, dasatinib did not affect neutrophil migration towards fMLP or IL-8 under those conditions (Fig 5F).

Taken together, though dasatinib inhibited two-dimensional neutrophil migration under static conditions and reduced neutrophil adhesion under static and flow conditions, it did not affect the migration of the cells under flow or in three-dimensional environments such as Transwell filters or Matrigel matrices.

**Recognition of innate immune ligands**

We next tested whether dasatinib affected neutrophil responses triggered by innate immune ligands of microbial origin (other than bacterial formyl peptides).

Zymosan, an extract of fungal walls, triggers various signal transduction pathways including Toll-like receptor and C-type lectin pathways and, when opsonized by serum proteins, may also engage complement- and Fc-receptors. Dasatinib inhibited the respiratory burst (Fig 6A) and the phosphorylation of ERK and the p38 MAP-kinase (Fig 6B) triggered by unopsonized zymosan. The effect of dasatinib on
normal serum-opsonized zymosan was slightly less pronounced (Figs 6A-B), especially in the case of ERK phosphorylation which was not sensitive to dasatinib. Neutrophil responses triggered by zymosan opsonized with heat-inactivated serum were similar to those triggered by unopsonized zymosan, suggesting that complement-mediated opsonization (and, possibly, concomitant release of complement fragments such as C5a) slightly reduces the sensitivity towards dasatinib.

We also tested the effect of dasatinib on neutrophil responses triggered by Toll-like receptor agonists. As shown in Fig 6C, dasatinib reduced gelatinase release triggered by the TLR2 agonist Pam3CSK4. Higher concentrations of dasatinib also partially reduced the activation of the p38 MAP-kinase by Pam3CSK4 and the highest concentration of the drug caused significant inhibition of p38 MAP-kinase activation triggered by ultrapure LPS (Fig 6D).

Taken together, dasatinib inhibited the responses of neutrophils to unopsonized zymosan but that effect was slightly attenuated by complement-mediated opsonization. Dasatinib also partially inhibited neutrophil functions triggered by TLR ligands, but that inhibition was primarily seen at very high concentrations of the drug.

**Effect on bacterial killing and phagocytosis**

We also aimed to test whether dasatinib affected antimicrobial activities of neutrophils. As shown in Fig 6E, dasatinib caused a modest reduction of neutrophil-mediated killing of serum-opsonized *S. aureus* or *E. coli* with IC50 values above 100 nM (Fig 6E). Dasatinib did not affect phagocytosis of serum-opsonized GFP-expressing *S. aureus* bacteria (Fig 6F), whereas the same response was strongly inhibited by 10 µM cytochalasin D (data not shown), indicating that we indeed tested an active phagocytosis process. Taken together, dasatinib does not have a major effect on the direct antimicrobial activities of neutrophils.

**Dasatinib inhibits leukocyte adhesion in whole serum**

All the above experiments were performed on neutrophils separated from their natural environment, in the absence of any serum proteins. We next tested whether dasatinib also inhibits neutrophils under more physiological conditions in the presence of whole serum. To this end, red blood cells were sedimented from freshly drawn human blood and the leukocyte-rich plasma supernatant was used in adhesion assays without any additional manipulation to preserve near-physiological conditions. The majority of adherent cells in this assay showed a typical neutrophil-like morphology (not shown).
As shown in Fig 7A, adding TNF, C5a, Pam3CSK4 or upLPS induced robust increase in leukocyte (neutrophil) adhesion to an FCS-coated surface and all those responses were practically completely blocked by dasatinib. In case of TNF stimulation, dasatinib caused a modest but statistically significant \((p = 0.013, n = 9)\) inhibition at as little as 50 nM concentration whereas in case of C5a stimulation, a robust and highly significant \((p = 0.00074; n = 6)\) inhibition was achieved by 100 nM dasatinib. In both cases, half-maximal inhibition was attained slightly above 200 nM dasatinib. Taken together, 50-100 nM dasatinib significantly inhibits neutrophil adhesion in the most sensitive assay systems and half-maximal inhibition is reached at around 200 nM concentration in the presence of whole serum.

Per os administration of dasatinib inhibits ex vivo adhesion of murine leukocytes

Our final aim was to test whether oral administration of dasatinib to experimental mice also affected neutrophil functions. That was performed by collecting peripheral blood two hours after oral dasatinib treatment, followed by the ex vivo analysis of leukocyte adhesion from leukocyte-rich plasma as described above. As shown in Fig 7B, ex vivo administration of TNF triggered robust leukocyte adhesion to an FCS-coated surface. That response was nearly completely blocked by prior oral administration of as little as 5 mg/kg dasatinib with a calculated IC\(_{50}\) value of 4.6 mg/kg. Those results indicate that oral administration of dasatinib inhibits leukocyte (likely primarily neutrophil) adhesiveness in experimental mice.

DISCUSSION

During the last several years, tyrosine kinases have emerged as major therapeutic targets in various malignant diseases\(^9\). Therapy of chronic myelogenous leukemia with the Abl kinase inhibitor imatinib became the first example of a tyrosine kinase inhibitor used in human therapy\(^40\). Later emergence of imatinib-resistant leukemia\(^41\) prompted the development of second-generation agents such as dasatinib which is able to inhibit imatinib-resistant BCR-Abl mutants\(^2\).

While dasatinib is able to suppress the proliferation of malignant hematopoietic cells, its effect on non-malignant cells is poorly understood. This is particularly true for neutrophils. The present study indicates that dasatinib exerts robust inhibitory effects on neutrophil responses triggered by cellular adhesion (Figs 1 and 2A-G) or immobilized IgG immune complexes (Fig 3), with IC\(_{50}\) values in the low nM range (often below 10 nM) in the most sensitive assay systems under serum-free conditions. On the other hand, neutrophil responses triggered by cytokines (Figs 2H-K), G-protein-coupled
receptor agonists (Fig 4) and innate immune ligands (Figs 6A-D), and direct antimicrobial functions of neutrophils (Figs 6E-F) were either modestly reduced or inhibited only at very high (1 µM) dasatinib concentration.

We also tested the effect of dasatinib on directed migration of neutrophils (Fig 5). Dasatinib blocked neutrophil migration in a two-dimensional Zigmond chamber assay and, as expected, reduced neutrophil adhesion both under static and flow conditions. However, dasatinib did not affect mechanotactic migration of cells that remained adherent under flow or the transmigration of neutrophils in three-dimensional settings such as Transwell filters or extracellular matrices. In our preliminary in vivo studies, dasatinib did not inhibit neutrophil accumulation during a thioglycollate-induced sterile peritonitis either (K. F. and A. M., unpublished observations). Though the reason for that discrepancy is unclear, the above results argue against a major effect of the drug on in vivo neutrophil migration. Though it would be tempting to speculate that the discrepancy is due to the requirement for integrins during two-dimensional but not three-dimensional migration of neutrophils, the fact that the Transwell migration (and likely the mechanotactic migration under flow) is dependent on β2-integrins argues against that possibility. It should also be mentioned that the discrepancy between defective adherent activation (Figs 1-2) and normal migration (Figs 5D-F) is in agreement with our prior conclusion that the two processes use different signal transduction pathways.

Since approx. 96% of dasatinib binds to serum proteins in vivo, we also tested the effect of the drug on leukocyte adhesion in whole serum. Dasatinib completely blocked leukocyte (primarily neutrophil) adhesion under those conditions with significant inhibition at 50-100 nM and IC₅₀ values around 200 nM in the most sensitive assay systems used (Fig 7A). We also administered dasatinib orally to experimental mice, followed by ex vivo analysis of the adhesion of their leukocytes in the presence of autologous serum. Oral administration of 5 mg/kg dasatinib practically completely blocked leukocyte adhesion under those conditions. That dose is near to or below doses previously used to treat various leukemia models in experimental mice.

The recommended dosage of dasatinib in human patients ranges from 70 mg once or twice daily to 100-140 mg once daily. The maximum dasatinib serum concentration following the most thoroughly tested 70 mg dose was reported in the range of 50-100 ng/ml (approx. 100-200 nM). Even higher serum concentrations are expected after administration of more than 70 mg dasatinib. There is significant variation in dasatinib plasma concentrations between individual patients and different ethnic groups. A recent study in pediatric patients reported dasatinib plasma concentrations in the range of 100-200 ng/ml (approx. 200-400 nM) and even
two cases of >240 ng/ml (approx. >480 nM) without dose-limiting toxicity. Those studies together suggest that plasma dasatinib concentrations significantly above 100-200 nM likely occur in certain dasatinib-treated patients, indicating that at least some of the effects on mature neutrophils likely occur during dasatinib treatment in human patients.

Dasatinib is a dual inhibitor of Abl and Src-family tyrosine kinases but it also inhibits additional tyrosine kinases such as c-Kit and the Eph family, and many of those kinases may be involved in neutrophil signaling. We can nevertheless speculate about how dasatinib inhibits neutrophils. While Abl-deficient mouse neutrophils showed normal integrin and Fc-receptor signaling, Src-family kinases were clearly indispensable for neutrophil activation by both integrins and Fc-receptors. The inhibitory effect of dasatinib on cellular tyrosine phosphorylation is very similar to that seen in Src-family-deficient neutrophils. Src-family kinases activate Syk downstream of integrins and Fc-receptors (unpublished observations) in neutrophils while Syk is required for signaling by both integrins and Fc-receptors (unpublished observations) in those cells. Since dasatinib strongly inhibited Syk activation following integrin (Fig 2E-G) and Fc-receptor (Fig 3F-G) ligation, a feasible explanation is that dasatinib inhibits Src-family-mediated activation of Syk, thereby blocking integrin and Fc-receptor signal transduction in neutrophils.

Drugs affecting immune cells (including neutrophils) may also attenuate antimicrobial immunity. Indeed, one of the side effects of dasatinib is the emergence of mild to moderate infections. The neutrophil compartment may be important during those side effects either because dasatinib may trigger myelosuppression and neutropenia or because of the direct effect of dasatinib on the antimicrobial functions of mature neutrophils. While our results presented in Fig 6 make it unlikely that therapeutic concentrations of dasatinib directly reduce the antimicrobial effect of neutrophils, the strong inhibition of adherent activation in the presence of antimicrobial compounds (Fig 1A) raises the possibility that dasatinib may reduce the inflammatory response required for antimicrobial immunity.

The dramatic effect of dasatinib on adhesion-mediated and immune complex-induced neutrophil functions also raises the possibility that dasatinib-related molecules may be effective in autoimmune or inflammatory diseases characterized by excessive neutrophil activation. That possibility is further emphasized by the significant inhibition of neutrophil-mediated in vivo models of inflammation (such as K/BxN serum-transfer arthritis or the Arthus reaction) by oral administration of dasatinib.
unpublished observations). Therefore, kinase inhibitors sharing structural similarities or overlapping target profiles with dasatinib may also provide clinical benefit in autoimmune or inflammatory diseases in the future.

ACKNOWLEDGMENT

We thank Edina Simon and Anna Tóth for expert technical assistance; Csaba Szántai-Kis, Eszter Illyés and István Varga for the HPLC-MS analysis; Csaba Tímár for help with bacterial killing assays; Erzsébet Ligeti and József Mandl for access to equipment; and William Nauseef for the GFP-expressing S. aureus strain. This work was supported by the Wellcome Trust (International Senior Research Fellowships No. 087782 to A. M.), the Hungarian Office for Research and Technology (Anyos Jedlik Award No. NKFP-A1-0069/2006 to A. M. and T. V.), the European Research Council (Starting Independent Investigator Award No. 206283 to A. M.), the Deutsche Forschungsgemeinschaft (grant SFB914/A2 to B. W.) and the European Union's FP7 Cooperation Program (TARKINAID project to A. M. and B. W.).

AUTHORSHIP CONTRIBUTIONS

K. F., T. N., T. V. and A. M. designed the work. K. F. and T. N. performed the majority of the experiments and analyzed the data. K. F., T. N. and A. M. interpreted the data and wrote the manuscript. R. P. and B. W. designed, performed, analyzed and interpreted the Zigmond chamber and flow chamber migration assays and wrote the relevant parts of the manuscript. A. M. supervised the project.

CONFLICT OF INTEREST DISCLOSURES

The authors declare that no financial conflict of interest exists.
REFERENCES


Figure legends

Figure 1
Dasatinib abrogates adhesion-dependent respiratory burst of human neutrophils
Human neutrophils pretreated with the indicated concentrations of dasatinib were stimulated with 20 ng/ml human TNF, 50 ng/ml human C5a, 1 µg/ml Pam3CSK4 or 1 µg/ml ultrapurified LPS (upLPS) while adherent to a fibrinogen-coated (Fbg; A and F) or FCS-coated (B) surface, by plate-bound antibodies against human CD18 (C), by plating them on a poly-RGD-coated surface (D) or by 100 nM PMA (E), followed by spectrophotometric measurement of superoxide release (A-E) or luminometric measurement of reactive oxygen production (F). Kinetic curves show mean and SD of representative experiments whereas dose-response curves show mean and SEM of percent response from 3-11 independent experiments. RLU, relative luminescence unit.

Figure 2
Dasatinib inhibits other adhesion-dependent responses but does not block TNF signaling
A-G, Human neutrophils pretreated with the indicated concentrations of dasatinib were stimulated with 20 ng/ml human TNF while adherent to a fibrinogen (Fbg) coated surface (A-E and upper part of G), or plated on a poly-RGD-coated surface (F and lower part of G), followed by microscopic observation (A) and quantification (B) of cell spreading, measurement of cell adhesion (C), assessment of lactoferrin release (D), direct analysis of total cellular tyrosine phosphorylation and phosphorylation of Syk Tyr352 by immunoblotting (E-F) or analysis of Syk phosphorylation by immunoprecipitation (IP) followed by immunoblotting for phosphotyrosine (PY) residues (G). H-K, Human neutrophils pretreated with the indicated concentrations of dasatinib were stimulated with TNF in suspension, followed by analysis of gelatinase release by in-gel zymography (H), flow cytometric analysis of expression (I) and activation (J) of CD11b, or phosphorylation of ERK and the p38 MAP-kinase by immunoblotting (K). Bar graphs in panels B-D show mean and SD of representative experiments, whereas dose-response curves in panels I-J show mean and SEM of percent response. Mean fluorescence intensity values of isotype control-stained samples were subtracted in panels I-J and the resulting fluorescence was expressed in percentage of that of the indicated samples. Each set of data was obtained from 3-9 independent experiments. The mean and SEM of phosphorylation of the various MAP-kinases at 10 nM, 100 nM and 1 µM dasatinib after subtraction of unstimulated control values corresponded to 101±18%, 94±17% and 56±8% (p38 MAP-kinase) and 89±31%, 85±26% and 89±20% (ERK) of that in the absence of dasatinib, respectively.

Figure 3
Dasatinib inhibits neutrophil functions triggered by immobilized immune complexes
Human neutrophils pretreated with the indicated concentrations of dasatinib were plated on a surface coated with immobilized IgG immune complexes (IC), followed by microscopic observation (A) and quantification (B) of cell spreading, measurement of respiratory burst (C), assessment of lactoferrin (D) and gelatinase (E) release, analysis of phosphorylation of total cellular proteins and the phosphorylation of the p38 MAP-kinase and Syk (Tyr352) by immunoblotting (F), or analysis of Syk phosphorylation by immunoprecipitation (IP) followed by immunoblotting for phosphotyrosine (PY) residues (G). Panels A, E and F show representative data from 3-4 independent experiments. The kinetic/bar graphs in panels B-D show mean and SD of representative experiments whereas dose-response curves in those panels show mean and SEM of percent response from 3-4 independent experiments.
Figure 4

*Dasatinib partially inhibits neutrophil functions triggered by G-protein-coupled receptors*

Human neutrophils pretreated with the indicated concentrations of dasatinib with or without 10 µM cytochalasin B (CB) were stimulated with 1 µM fMLP (A-E), 100 ng/ml human IL-8 (F-H), 50 ng/ml C5a (I), or 50 ng/ml LTB4 (J) followed by measurement of the respiratory burst (A), lactoferrin release (B), flow cytometric analysis of the expression (C, F) and activation (D, G) of CD11b, or assessment of phosphorylation of the ERK or p38 MAP-kinases by immunoblot and analysis of gelatinase release by in-gel zymography (E, H-J). The kinetic/bar graphs in panels A-B show mean and SD of representative experiments whereas dose-response curves in those panels and bar graphs in panels C-D and F-G show mean and SEM of percent response. Mean fluorescence intensity values of isotype control-stained samples were subtracted in panels C-D and F-G and the resulting fluorescence was expressed in percentage of that of the indicated samples. Each set of data was obtained from three to five independent experiments. Panels E and H-J are representative of three independent experiments. The mean and SEM of phosphorylation of the various MAP-kinases in the stimulated samples after subtraction of unstimulated controls in the presence of 10 nM, 100 nM and 1 µM dasatinib was 103±10%, 99±14% and 77±17% (E; p38 MAP-kinase), 99±9%, 94±6% and 100±2% (E; ERK), 90±17%, 53±5% and 16±5% (H; p38 MAP-kinase), 123±39%, 112±11% and 169±42% (H; ERK), 102±52%, 75±27% and 24±12% (I; p38 MAP-kinase), 98±5%, 98±12% and 195±55% (I; ERK), 113±53%, 79±29% and 62±60% (J; p38 MAP-kinase) and 89±14%, 173±32% and 236±49% (J; ERK) of that in the absence of dasatinib.

Figure 5

*The effect of dasatinib on neutrophil migration*

A-B, human neutrophils pretreated with the indicated concentrations of dasatinib were allowed to migrate towards 10 µM fMLP (A) or 1 µg/ml human IL-8 (B) on a fibrinogen-coated coverslip in a Zigmond chamber assay. Representative single-cell tracks, rose diagrams of direction of movement and the results of quantitative analysis are shown. Bar graphs show mean and SD of representative experiments whereas dose-response curves show mean and SEM of percent response from 263-476 cells from 3-7 independent experiments. C-D, Adhesion (C) and migration (D) of neutrophils in the presence of 1 µM fMLP in a flow chamber assay (all samples were treated with fMLP). Panel C shows the number of adherent cells per field of view (FOV). Panel D shows migration characteristics of cells that remained adherent through the entire 10-min flow period. Data in panels C-D show mean and SEM from 5 independent experiments. E-F, cells were allowed to migrate towards 100 nM fMLP or 10 ng/ml IL-8 through FCS-coated Transwell filters (E) or a Matrigel matrix (F). Data in panels E-F show mean and SEM from 4-5 independent experiments.

Figure 6

*The effect of dasatinib on recognition of innate immune ligands and antimicrobial activity*

Human neutrophils pretreated with the indicated concentrations of dasatinib were stimulated with 10 mg/ml zymosan (Zym) that was opsonized with normal or heat-inactivated (HI) human serum or left unopsonized (A-B), 1 µg/ml Pam3CSK4 (Pam3; C-D) or with 1 µg/ml ultrapurified LPS (upLPS; D), followed by assessment of respiratory burst (A), ERK and p38 MAP-kinase phosphorylation (B, D) or gelatinase release (C). E-F, Killing of S. aureus or E. coli (E) and phagocytosis of GFP-expressing S. aureus (F) by human neutrophils pretreated with the indicated concentrations of dasatinib. Kinetic curves in panels A and E show mean and SD of representative experiments whereas dose-response curves in those panels show mean and SEM of percent response from three to six independent experiments. Panels B-D show representative results from three independent experiments. Panel F shows mean and SEM of
Mean fluorescence intensity (MFI) values from 3-4 independent experiments, after subtraction of samples without bacteria at the 0 time point and expressed in percent of the indicated sample. The mean and SEM of phosphorylation of the various MAP-kinases in the stimulated samples after subtraction of unstimulated control values in the presence of 10 nM, 100 nM and 1 µM dasatinib was 62±27%, 5±2% and 5±3% (B; unopsonized Zym; p38 MAP-kinase), 81±23%, 43±9% and 52±26% (B; unopsonized Zym; ERK), 42±19%, 7±7% and 5±5% (B; HI serum-opsonized Zym; p38 MAP-kinase), 56±24%, 37±16% and 72±38% (B; HI serum-opsonized Zym; ERK), 73±12%, 9±4% and 4±4% (B; normal serum-opsonized Zym; p38 MAP-kinase), 84±1%, 69±2% and 89±11% (B; normal serum-opsonized Zym; ERK), 160±38%, 138±57% and 55±24% (D; Pam3CSK4 and 154±31%, 72±24% and 15±14% (D; upLPS) of that in the absence of dasatinib.

**Figure 7**

*Dasatinib inhibits adhesion of human and mouse leukocytes in whole serum*

A, Leukocyte-rich human plasma pretreated with the indicated concentrations of dasatinib was incubated with 20 ng/ml human TNF, 100 ng/ml human C5a, 1 µg/ml Pam3CSK4 or 1 µg/ml ultrapurified LPS (upLPS) on an FCS-coated surface for 30 min followed by determination of cell adhesion. B, Leukocyte-rich mouse plasma collected two hours after oral administration of the indicated doses of dasatinib was stimulated with 50 ng/ml murine TNF on an FCS-coated surface for 30 min, followed by determination of cell adhesion. Bar graphs show mean and SD of representative experiments, whereas dose-response curves show mean and SEM of percent response from 3-9 independent experiments.
Figure 2

A

B

C

D

E

F

G

H

I

J

K

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Figure 5

A

B

C

D

E

F

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Dasatinib inhibits pro-inflammatory functions of mature human neutrophils

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