Accepted Manuscript

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PII: S0301-472X(15)00037-5

DOI: [10.1016/j.exphem.2015.01.008](https://doi.org/10.1016/j.exphem.2015.01.008)

Reference: EXPHEM 3230

To appear in: *Experimental Hematology*

Received Date: 8 October 2014

Revised Date: 19 January 2015

Accepted Date: 21 January 2015

Please cite this article as: Oksvold MP, Duyvestyn JM, Dagger SA, Taylor SJ, Forfang L, Myklebust JH, Smeland EB, Langdon WY, The targeting of human and mouse B lymphocytes by dasatinib, *Experimental Hematology* (2015), doi: 10.1016/j.exphem.2015.01.008.

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The targeting of human and mouse B lymphocytes by dasatinib

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Keywords: B cells, BCR, B cell development, Btk, Dasatinib

List of abbreviations: B cell receptor (BCR), tyrosine kinase inhibitors (TKIs), chronic myeloid leukemia (CML).

Word count: 4313

Abstract

Dasatinib inhibits BCR-ABL, Src and other tyrosine kinases. Few studies have addressed the impact of dasatinib on normal blood cells, especially in vivo. Here we show that dasatinib leads to a reduced number of human CD19⁺ peripheral B cells, due to a strong induction of apoptosis. In contrast, no similar effect on T-cell viability was observed. However, dasatinib induced a comparable broad inhibition of the early events of B and T cell receptor signaling. Furthermore, dasatinib was shown to be a more pronounced inhibitor of both basal and BCR-induced activity of Btk and PLC γ 2 compared to the more specific Btk inhibitor ibrutinib. Human progenitor B cells from the pre-B stage were sensitive to dasatinib. In an in vivo murine model, dasatinib reduced B-lineage cells in the BM with a marked effect on the pre-B subpopulation. Dasatinib led to a reduced spleen size, with a loss of large immature transitional IgM⁺/IgD⁻ B cells and a reduction in germinal center B cells. Dasatinib caused a marked loss of thymocytes without affecting myeloid lineage cells or hematopoietic progenitors. This study reveals important side effects of dasatinib with specific loss of activated B and thymocyte populations which may have an impact during long-term treatment.

Introduction

Tyrosine kinase inhibitors (TKIs) represent the first group of targeted molecular drugs that have entered into frequent clinical use for the treatment of cancer, in particular blood, kidney and lung cancer. For Philadelphia chromosome-positive chronic myeloid leukemia (CML) and acute lymphoblastic leukemia, TKIs have significantly improved patient outcomes [1-5] with imatinib generally being used as a first-line treatment of these malignancies. The second-generation TKI dasatinib, a small molecule kinase inhibitor originally introduced to target SRC kinase, was also found to bind Abl with an affinity much higher than imatinib [6]. Dasatinib is now widely used in imatinib resistant or intolerant patients where it has further improved the survival for CML patients [7-9]. Dasatinib induces higher and more rapidly complete cytogenic responses compared to imatinib, and it has been suggested that administration of dasatinib as the initial therapy may improve responses in patients with newly diagnosed chronic-phase CML.³ Since dasatinib inhibits Lyn kinase and the understanding that Lyn kinase might be critical for chronic lymphocytic leukemia (CLL) survival [10], studies of dasatinib in CLL have been initiated [11-12]. Dasatinib has also been suggested as possible treatment for certain B cell lymphoma malignancies due to its inhibition of Lyn and Btk [13-14].

Due to dasatinib's broad inhibitory effect on a number of different kinases [15-18] one would expect more side effects related to this drug. However, although well tolerated in most patients, dasatinib has been reported to induce side effects, such as pleural effusion, arthralgia, fever and cytopenias [19-20]. It has also been reported that some patients treated with dasatinib are prone to severe infections indicating immunosuppressive activity of the drug [21-22] although this was not confirmed in a larger patient cohort [3].

The wide use of dasatinib and the possibility of more extensive use of this drug in the future warrants further investigation of its effects. Few studies have addressed the impact of dasatinib on normal blood cells, especially in vivo, as the majority of studies have focused on effects on malignant cells. In this study we investigated the effects of dasatinib on normal human B lymphocytes derived from peripheral blood and B cell precursors from the BM, in addition to in vivo studies of B and T cells in mice. We demonstrated that dasatinib induced apoptosis in human CD19⁺ peripheral B cells, and that precursor B cells in the BM from the pre-B cell maturation stage are affected. In vivo studies of the effects of dasatinib on the hematopoietic system of mice showed that dasatinib specifically targets immature B-lineage cells and thymocytes, but does not affect progenitors or the myeloid lineage.

Results

Dasatinib induces apoptosis in human CD19⁺ peripheral blood B cells

Purified human CD19⁺ B cells from peripheral blood were cultured together with CD40L in the presence or absence of dasatinib. A concentration of 128 nM dasatinib was chosen as it is equivalent to the maximum serum concentration of dasatinib observed in CML patients using FDA-approved doses [23]. Dasatinib exposure led to a time-dependent increase in cell death of the CD19⁺ B cells. This effect was significant after 3 days of treatment, and was maintained after 4 and 5 days of exposure with 33% and 44% dead cells in the presence of dasatinib vs. 6% and 9% in controls, respectively (Fig. 1A). In contrast, the viability of IL-2 stimulated CD4⁺ and CD8⁺ peripheral blood T cells was not significantly affected by dasatinib treatment (Fig. 1B). To further characterize dasatinib-induced cytotoxicity, we analyzed the induction of apoptosis in human CD19⁺ B cells and CD4⁺ and CD8⁺ T cells from blood by TUNEL staining. After 5 days, a clear increase of TUNEL⁺ CD19⁺ cells was detected in the dasatinib treated cells (81%, vs 11% in untreated control cells) demonstrating that dasatinib potently induced apoptosis (Fig. 1C). A similar induction of apoptosis was not seen in the T cells.

Inhibition of BCR and TCR signaling with dasatinib

To further understand the selective apoptosis-inducing effect of dasatinib in B cells, but not T cells, we investigated the impact of dasatinib on the activation of B cell receptor (BCR) and T cell receptor (TCR) signaling in human PBMCs gated for CD20 and CD5, respectively. Phosphorylation of Syk, Btk, BLNK, PLC γ , MAPK ERK1/2 and p70 S6 kinase (pS6) was monitored in unstimulated and goat anti-human IgM F(ab')₂ stimulated CD20⁺ PBMCs (Fig. 2A-B). Exposure to dasatinib reduced the levels of pBtk and pPLC γ , suggesting basal activity

of these in B cells (Fig. 2A). Upon stimulation with goat anti-human IgM F(ab')₂ (aBCR), CD20⁺ B cells showed increased levels of pSyk, pBtk, pBLNK, pPLC γ , pERK1/2 and pS6 (Fig. 2A). Pre-treatment with dasatinib completely blocked this activation, with the exception of pS6 (Fig. 2A-B). To examine dasatinib effects on TCR activation, PBMCs were pretreated with dasatinib for 1 h and stimulated with anti-CD3/CD28 (Fig. 2C-D). Upon stimulation with anti-CD3/CD28, the CD5⁺ T cells showed increased pCD3 ζ , pZAP-70, pPLC γ and pSLP76 (Fig. 2C), which was completely abrogated by pre-treatment with dasatinib (Fig. 2C). In order to see if there were differences in the dasatinib-induced inhibition of basal Btk and Itk (homologue to Btk) phosphorylation between B and T cells, respectively, we analyzed CD20⁺ and CD5⁺ PBMCs exposed to dasatinib. Dasatinib inhibited the basal phosphorylation of both Btk and Itk, but with a significantly more pronounced effect on Btk (Fig. 2E). In summary, dasatinib inhibited BCR-induced signaling in B cells, but also TCR-induced signaling in T-cells. However, dasatinib significantly reduced the level of pBtk in unstimulated B cells.

Dasatinib is a more potent inhibitor of Btk and PLC- γ than ibrutinib

Since dasatinib reduced the basal level of pBtk in B cells, we next compared the effects of dasatinib with the Btk inhibitor ibrutinib on basal- and BCR-induced signaling. Btk is a downstream mediator of BCR signaling, is not expressed by T cells [24], and has been well characterized for its activation of PLC- γ 2 [25-27]. Pre-treatment with ibrutinib or dasatinib revealed dasatinib to be a markedly more potent inhibitor of anti-IgM induced activation of Syk, Btk and PLC- γ 2 (Fig. 3A). The more pronounced effect of dasatinib versus ibrutinib on BCR signaling was further illustrated by these inhibitors ability to abrogate B-cell viability. Culturing CD19⁺ B cells from peripheral blood with ibrutinib or dasatinib clearly showed that

dasatinib has a more pronounced negative effect on viability compared to ibrutinib treated cells (49% cell death vs. 24%, respectively; Fig. 3C). This was confirmed by TUNEL staining, demonstrating that dasatinib represents a more potent inducer of apoptosis compared to ibrutinib (61% TUNEL⁺ vs. 31% for ibrutinib; Fig. 3D-E).

We next tested the potential effects of dasatinib and ibrutinib in the presence of CD40L. CD40L induced activation of the NF- κ B pathway as demonstrated by reduced levels of total I κ B α and induced phosphorylation of p65 NF- κ B, as well as increased pERK1/2 (Supplementary Figure 1A-B). Preincubation with dasatinib or ibrutinib did not abrogate the CD40L-induced signaling. Furthermore, CD40L-induced activation of the non-canonical NF- κ B pathway involving RelB, was not affected by dasatinib pretreatment (Supplementary Figure 1C). Dasatinib reduced, as previously shown in Fig. 2, the basal levels of pBtk (Supplementary Figure 1C), and this was maintained after 24 h (data not shown). In summary, our results indicated that the effect of dasatinib on B cell viability was not caused by inhibition of the major CD40 signaling pathway directly. However, a prolonged abrogation of the basal Btk activity may be an important cause of the negative impact on normal peripheral blood B cells.

Since the T cells in our viability experiment (Fig. 1B) were cultured with IL-2, we analyzed if IL-2 signaling was affected by dasatinib. IL-2 induced activation of STAT5 was not inhibited in dasatinib treated CD5⁺ T cells from PBMCs (Supplementary Figure 2).

Dasatinib reduces human B cell numbers in the BM from the pre-B stage

In order to identify the stages in B cell development where dasatinib has an impact, we studied the viability of different B cell progenitor populations from human BM and mature B cells following exposure to dasatinib. Purified human CD19⁺ B cells from BM were stained and FACS-sorted into pro-B cells (CD19⁺CD34⁺IgM⁻), pre-B cells (CD19⁺CD34⁻IgM⁻), and immature B cells (CD19⁺CD34⁻IgM⁺). The gating strategy for the different populations is shown (Fig. 4A). In addition we sorted mature B cells (CD19⁺CD10⁻) derived from blood that is present in the BM aspirates. After three days in co-culture with stromal cells we observed a significant decrease in viability for the pre-B, and mature B cell populations exposed to dasatinib, as shown by PI staining (Fig. 4B). In contrast, pro-B cells and immature B cells were not significantly affected by dasatinib treatment. This suggests that the B-cell precursors at the maturation step between pro-B and pre-B cells are dependent on the activity of kinases, either directly or indirectly targeted by dasatinib.

Dasatinib dosing of mice reduces B-lineage cells in the BM

To examine the *in vivo* effects of dasatinib on B-lineage cells, we dosed C57BL/6 mice once daily for 4 weeks with 30 mg/kg of dasatinib or vehicle. This dose was chosen as it is comparable to the daily dose commonly used for treating CML patients [28,29]. Analysis of BM cells by flow cytometry revealed that B-lineage cells were profoundly affected, with a significant decrease in the proportion of both IgM⁺ and IgM⁻ cells within the B220⁺ population (Fig. 5A-B). In contrast, the percentage of CD11b⁺/Gr-1⁺ myeloid lineage cells was increased (Fig. 5C-D).

To determine which cells within the B220⁺ IgM⁻ population were most affected by dasatinib, we examined the expression of CD24 and CD43, markers that define PrePro-B, Pro-B and Pre-B stages of differentiation [30]. The analysis revealed that dasatinib significantly reduced the proportion of Pre-B cells, a subpopulation of B220⁺ IgM⁻ cells that are characterized by CD24^{hi} and CD43^{lo} expression (Fig. 5E-F). Concomitant with this, a corresponding increase in the proportion of Pro-B and PrePro-B cells was observed.

Since dasatinib is well characterized for its inhibition of the c-Kit receptor tyrosine kinase we also examined its effects on c-Kit⁺ hematopoietic progenitor and stem cell populations. The gating strategies for the progenitor and stem cell populations are shown (Supplementary Fig. 3 A and B). The analysis by flow cytometry showed no significant reduction in any of the c-Kit⁺ populations examined, including common lymphoid progenitors (CLPs; Fig. 5G). The absence of long-term effects on hematopoietic progenitors and stem cells is surprising given dasatinib's ability to inhibit c-Kit and the requirement of these cells for c-Kit signaling [31]. However, we did observe a slight but statistically significant reduction in c-Kit expression on lineage negative, Sca-1⁺ c-Kit⁺ (LSK) cells and long-term hematopoietic stem cells (LT-HSCs) from dasatinib dosed mice when single parameter plots were compared (Supplementary Fig. 3C). These observations suggest further investigation of the effects of dasatinib on hematopoietic stem and progenitor cells is warranted. When we examined serum from mice after 4 weeks of dosing a significant increase in the c-Kit ligand [i.e, stem cell factor (SCF)] was observed (Fig. 5H), suggesting a compensatory response that may provide a possible explanation as to why no large loss of c-Kit⁺ cells was evident.

Dasatinib targets immature B cells in the spleen

Examination of the effects of dasatinib on spleens from C57BL/6 mice revealed a small but statistically significant decrease in size (Fig. 6A). Analysis of splenic B cells by flow cytometry showed that the effect of dasatinib on spleen size may be at least in part due to a significant loss of large immature transitional $\text{IgM}^+ \text{IgD}^-$ cells (i.e. T1 B cells) and $\text{IgM}^+ \text{IgD}^+$ (T2) B cells (Fig. 6B-C). This finding is consistent with transitional B cells being large activated cells (Fig. 6D) requiring tonic B cell receptor signaling and SFK activation for survival [32-33]. The loss of transitional B cells was accompanied by a proportional increase in small, quiescent follicular B cells that do not require B cell receptor signaling and SFK activation for their maintenance (Fig. 6B-D). The effect of dasatinib on germinal center (GC) B cells was also examined since they are highly proliferative and dependent on Lyn kinase signaling [34-36]. Dasatinib was associated with a significant reduction of B220^+ cells that express the GC markers CD95 and GL7 (Fig. 6E), a finding consistent with their highly proliferative phenotype and reliance on SFK activation. In contrast, T cells and myeloid cells in the spleen were not significantly affected by dasatinib (Fig. 6F). To examine the effect of dasatinib *in vitro* on murine B cells we cultured purified splenic B cells from a C57BL/6 mouse for three days with CD40L and in the presence or absence of 128 nM dasatinib. We found a similar effect to that observed with human B cells where dasatinib induced a marked induction of cell death (Supplementary Fig. 4).

Dasatinib causes a marked loss of thymocytes in mice

The requirement of SFK signaling to promote the survival of double positive (DP) thymocytes and enable their positive selection to single positive (SP) CD4 and CD8 cells is well characterized [37]. This requirement was clearly illustrated when we analyzed thymocytes from mice dosed for 4 weeks with dasatinib or vehicle. We found that dasatinib

markedly reduced thymocyte numbers (Fig. 7A), and this reduction was seen in all populations, i.e. double negative (DN), DP and SP populations (Fig. 7B). Consistent with immature DN cells being less reliant on SFK signaling we found that their proportion was increased relative to vehicle treated mice (Fig. 7C-D), although there was a loss in total numbers (Fig. 7B). To the best of our knowledge no previous studies have examined the effects of dasatinib on thymocytes in an animal model.

Dasatinib does not reduce the numbers of peripheral white blood cells in mice

Mice were bled three days before dosing commenced (Pre-Tx), and after 4 weeks of dosing with dasatinib or vehicle. Analysis by Hemavet differential counting showed that dasatinib did not reduce total white blood cell (WBC) counts (Supplementary Figure 5A), or alter the percentages of lymphocytes or neutrophils (Supplementary Figure 5B-C). Further analysis by flow cytometry showed no change in the proportion of B cells, T cells or CD11b⁺ myeloid cells (Supplementary Figure 5D-F, respectively). Thus, in contrast to the effects of dasatinib on pre-B and immature B cells in the BM, on transitional B cells in spleen, and thymocytes in the thymus, dasatinib did not reduce the numbers of B and T cells in peripheral blood. In addition, dasatinib dosing did not alter the numbers of platelets, red blood cells, or hemoglobin levels (data not shown).

Discussion

Dasatinib represents a widely used tyrosine kinase inhibitor for the treatment of CML and acute lymphoblastic leukemia, and recent clinical trials for the treatment of CLL are promising. Therefore it is surprising that few studies have addressed the effect of dasatinib on normal immune cells *in vivo* and *in vitro*. Recently, the inhibitory effects of dasatinib on B-cell activation and the induction of humoral immune responses was recognized [38]. A significant decrease in the frequencies of memory B-cell subsets was observed when a state of minimal residual disease was achieved after treatment of CML, suggesting a direct effect of dasatinib on B cells. Furthermore, we recently found that dasatinib reduced B-cell numbers in c-Cbl RING finger mutant mice that developed a myeloproliferative disease, however the effects on normal murine B cells were not examined in detail [39]. Here we show that dasatinib markedly affects B lymphocytes from healthy humans and mice. In humans, both peripheral CD19⁺ blood B cells and pre-B cells and immature B cells from BM were found to be highly sensitive to dasatinib-induced cell death. The potent effect of dasatinib on BCR signaling was clearly shown by its inhibition of anti-BCR induced activation of Syk and its downstream effectors PLC- γ 2, pS6 and ERK1/2 in primary human peripheral B cells (Fig. 2A-B). Previous studies with dasatinib have reported very efficient inhibition of BCR-induced activation of both Lyn in primary mantle cell lymphoma cells [13] and ERK1/2 and Akt in CLL cells [40]. Peripheral B cells in culture are dependent on CD40 signaling for survival. However, we were unable to link the reduced viability observed in dasatinib treated peripheral B cells to effects on the CD40 signaling pathway. The impact of dasatinib on the survival of pre-B cells could be explained in part by its effect on Btk which we show to be markedly inhibited by dasatinib. However, Btk is activated by SFK Lyn in addition to Syk [41-43]. Furthermore, dasatinib can directly inhibit Btk autophosphorylation and activity through

specific binding to the gatekeeper residue [16]. In addition, it has been reported that Btk is not required in pro-B and large pre-B cells, but is critical for the transition from pre-B cells and survival of immature B cells [24]. Notably, it has previously been shown that Btk is constitutively activated in pre-B cells [44].

We also showed that anti-CD3/CD28 activation of its downstream signaling molecules ZAP70, PLC- γ 1 and SLP76 was markedly inhibited by dasatinib (Fig. 2B). It has earlier been shown that dasatinib abrogates the SFK Lck and its downstream effectors in WEHI7.2 cells [45]. A similar effect of dasatinib was reported in TCR transfected COS-7 cells and human primary T cells stimulated with PHA [46]. However this is the first report to show the effects of dasatinib on TCR signaling in normal human T cells.

Despite dasatinib's potent effect on TCR signaling, the viability of peripheral blood T cells in culture with IL-2 and dasatinib was comparable to control cells. It has previously been shown that the activity of IL-2 inducible T-cell kinase (Itk), the Btk homologue expressed in T cells, is not affected by dasatinib [16]. Of note we observed inhibition of basal Itk activity in peripheral T cells, however the inhibition of basal Btk activity was more pronounced.

Furthermore, IL-2- induced signaling through STAT5 was not affected by dasatinib pretreatment in peripheral T cells. Therefore, a possible explanation for the different effects of dasatinib on human peripheral B and T cells could be that B cells have a greater dependence on SFKs for survival, and/or the more profound inhibition of Btk in B cells could also play a major role.

Dosing of mice with dasatinib resulted in a significant loss of pre-B cells in the BM, and of large immature B cells and GC B cells in the spleen. Importantly, these populations are dependent on SFK signaling for survival, which is efficiently blocked by dasatinib. The profound effect of dasatinib on Pre-B cells is consistent with their requirement for Pre-B cell receptor engagement and the activation of SFKs [47]. Similarly dasatinib had a marked effect in reducing thymocyte numbers, which is likely to be mediated by dasatinib's inhibition of SFKs Lck and Fyn. In contrast, peripheral T and B cells in the blood, which are not exposed to strong TCR and BCR survival signals, were not affected.

Surprisingly we found no effects of dasatinib on myeloid lineage cells, a result that contrasts the inhibitory effects when myeloid cells are driven by BCR-ABL. It is well known that SFKs are active in myeloid lineage cells so the reason for the inability of dasatinib to suppress this population remains to be determined. A possibility is through compensatory responses by the induction of myeloid growth factors, although we did not find an increase in either GM-CSF or G-CSF in the serum of dasatinib treated mice (data not shown). Dasatinib also showed no obvious effects on c-Kit⁺ progenitors and stem cells, an unexpected result given dasatinib's inhibition of c-Kit kinase activity. However it is likely that dasatinib's inhibition of c-Kit is compensated by the induction of SCF which we found to be significantly elevated in the serum of dasatinib treated mice. Further characterization of this response by investigating the kinetics of SCF induction in the BM will be of interest.

In conclusion, treatment with dasatinib is most strikingly associated with the loss of activated B lymphocytes. Furthermore, dasatinib caused a marked loss of thymocytes. Thus the profound effects of dasatinib on lymphocytes and thymocytes may adversely affect patient

immunity. This warrants further studies given the expanding use of dasatinib as an anti-cancer treatment.

Materials and Methods

Human samples

B and T cells were isolated from buffy coats collected from anonymous, healthy donors at the Blood Bank in Oslo and BM aspirates were from healthy adult volunteers. All volunteers were provided written informed consent, validated by the Regional Ethics Committee (approval no S-03280 and S-90128, respectively), in accordance with the Declaration of Helsinki. B and T cells were isolated using positive selection with anti-CD19 and -CD4/-CD8 Dynabeads (Life Technologies, Oslo, Norway), respectively, followed by treatment with the corresponding DETACHaBEADs as described previously [48]. Peripheral blood mononuclear cells (PBMCs) and cells from BM aspirates were isolated using Ficoll-Hypaque density gradient sedimentation.

B cells were cultured in RPMI-1640 (PAA Laboratories, Austria) supplemented with 10% FCS (Lonza), penicillin and streptomycin, and added pre-incubated CD40L + enhancer for ligand (1 µg/mL; Enzo Life Sciences, NY). T cells were cultured in CellGro DC medium (CellGenix) with IL-2 (Chiron).

Mice

Male C57BL/6 mice were purchased from the Animal Resource Centre (Canning Vale, Western Australia). The mice were aged ~16 weeks when dosing commenced and all mouse

experiments were performed in accordance with the regulations of the Animal Ethics Committee at the University of Western Australia (approval 100/1169 and 100/786). Mice were dosed once daily with 0.2 ml of either dasatinib (30 mg/kg), or citrate buffer vehicle, by oral gavage using 20G 1.5 inch feeding needles (Braintree, MA).

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Dasatinib and ibrutinib for studies in human cells were obtained from Selleckchem (Houston, TX) and dissolved in DMSO. Dasatinib was obtained from LC Laboratories (Woburn, MA). Stock solutions were prepared freshly each week in 80 mM citric acid, pH 2.1 and diluted each day in citrate buffer pH 3.1 for dosing.

Phospho-specific flow cytometry

Phospho-specific flow cytometry with barcoding with Pacific blue dye, followed by staining with anti-CD5 and anti-CD20 followed by a panel of phospho-specific antibodies was performed as described previously [49]. Briefly, fixed and permeabilized cells were barcoded with different concentrations of Pacific blue dye, before staining with anti-CD5 and anti-CD20 followed by a panel of phospho-specific antibodies as indicated. Following antibodies were applied: CD5-PE-Cy7, CD20-PerCP-Cy5.5, pPLC γ 1/pPLC γ 2-Ax488, pSYK/ZAP-70-Ax647, pSFK-Ax488, pCD3 ζ -Ax647, pSLP76-Ax488, pErk1/2-Ax488, pBtk-Ax647, pSTAT5-Ax647, pBLNK-Ax647 and pNF- κ B p65 (BD Biosciences), and pp70 S6-Ax647, I κ B α -Ax647, pRelB-PE and pSAPK/JNK-AX647 (Cell Signaling Technologies). Flow cytometric analysis and data collection was performed on a FACSCantoII using FACSDiva software (BD Biosciences). Further analysis was performed using Cytobank software.

Cell death and apoptosis staining

Cells were stained with 5 µg/mL propidium iodide (PI; Life Technologies) or fixed in PFA (1% in PBS, 10 min at RT) and methanol (10 min at -20°C) before TUNEL staining (Cell death detection kit; Roche, Switzerland) and analysis by flow cytometry.

Analysis of peripheral blood, spleen and BM cells in mice

Blood was collected from the tail vein and differential cell counts determined using a Hemavet HV950FS blood analyzer (Drew Scientific, Waterbury, CT). BM cells were taken from tibias and femurs of each mouse [39]. Blood, BM and spleen cells were analyzed by flow cytometry (FACSCanto) using monoclonal antibodies and procedures described below. Data was collected using FACSDiva software and analyzed using FlowJo software (Tree Star, Ashland, OR).

Flow cytometry antibodies for mouse studies

All antibodies were from BD Biosciences, except where noted otherwise. CD3-biotin, CD11b-FITC -PE -PE-Cy7 -biotin, CD16/32-PE, CD19-PE, CD24-PE-Cy7, CD43-PE, CD48-FITC, CD95-PE, CD150-PE-Cy7, c-Kit-APC -FITC (eBioscience), Sca-1-PE – PE-Cy7 (eBioscience), B220-biotin, FLT3-PE (eBiosciences), Gr-1-FITC -biotin, IgM-APC, IgD-FITC (eBioscience), IL-7R-APC -biotin, TER119-biotin, GL7-FITC and TCR-APC. Cells incubated with biotinylated antibodies were treated with streptavidin conjugated with APC-Cy7 (BD Biosciences). Long-term hematopoietic stem cells (LT-HSCs) were identified as CD150⁺, CD48⁻ LSK cells, multipotent progenitors (MPPs) as FLT3⁺ LSK cells, common

lymphoid progenitors (CLPs) as Lin⁻, IL-7R⁺ Sca-1^{lo} c-Kit^{lo}. Pre-B, Pro-B and PrePro-B cells were determined by CD24 and CD43 staining of B220⁺, IgM⁻ BM cells. Germinal center B cells in the spleen were identified by the expression of CD95 and GL7.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Serum levels of stem cell factor were determined using Quantikine® immunoassay kits as recommended by the manufacturer's directions (R&D Systems).

Statistical analyses

To validate significance we used unpaired or paired two-sided Student's *t*-tests (Prism 5, GraphPad Software). *P* values less than 0.05 were considered statistically significant. All statistical data are presented as mean ± SEM.

Acknowledgements

We thank Nomdo Westerdal for the cell sorting and Dag Josefsen and Yngvar Fløisand for help with the BM aspirates. This work was funded by RCN, NCS, OUS Radiumhospitalet, NHMRC Project Grants 572516 and 634414, the Cancer Council of WA, the Medical and the Health Research Infrastructure Fund and the L.T Thean Memorial Scholarship.

Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest.

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Figure legends

Figure 1. Dasatinib induces apoptosis in human peripheral B cells. (A) CD19⁺ peripheral blood B cells were cultured with or without dasatinib (das, 128 nM) for 2-5 days, exposed to PI and cell death (PI⁺) was analyzed by flow cytometry. Shown is mean $\pm$ SEM (n=3-4), ^{###}*P* < 0.0004, ^{***}*P* < 0.0007, ^{**}*P* < 0.0086. (B) Human CD4⁺/CD8⁺ peripheral T cells were cultured in the presence of IL-2 (20 U/ml) with or without dasatinib (das, 128 nM) for 2 and 5 days, exposed to PI and cell death was analyzed by flow cytometry. Shown is mean $\pm$ SEM (n=3). (C) CD19⁺ B cells and CD4⁺ and CD8⁺ T cells were cultured $\pm$ dasatinib (128 nM) for 4 days, fixed and permeabilized, and then subjected to TUNEL assay, and analyzed by flow cytometry. One representative experiment (left) and average data for TUNEL⁺ cells are shown (right; mean $\pm$ SEM, n=3, **P* < 0.0115).

Figure 2. Dasatinib abrogates downstream BCR- and TCR signaling in human peripheral B and T cells. (A) Anti-IgM induced activation of the BCR signaling pathway with or without pretreatment with dasatinib (das) was determined in B cells by phosphoflow cytometry. Human MNCs from blood were preincubated with or without dasatinib (128 nM) for 1 h, before stimulation with anti-IgM F(ab')₂ for 4 min. Fixed and permeabilized cells were barcoded with pacific blue, collected in 1 tube per donor and then stained with anti-CD5/-CD20 in addition to the specified phospho specific mAb. Shown is heat map of median fluorescence intensity (MFI) of phospho-proteins relative to unstimulated controls (one representative experiment). (B) Relative levels of pSyk, pBtk, pPLC γ and pERK1/2, as normalized to unstimulated cells (control) (mean $\pm$ SEM, n=3). ^{**}*P* < 0.0067, *P* < 0.0018, *P* < 0.008 for pSyk, pBtk and pPLC γ , respectively (stim = stimulated, d + s = dasatinib + stimulated). (C) Anti-CD3/-CD28 induced activation of the TCR signaling pathway with or

without pretreatment with dasatinib (das) was determined in T cells by phosphoflow cytometry. Human CD4⁺/CD8⁺ cells were isolated from blood and treated with or without dasatinib (128 nM) for 1 h before stimulation or not with anti-CD3/anti-CD28 for 3 min. Fixed and permeabilized cells were treated as described in A) and stained with the specified phospho-specific mAbs. Shown is heat map of MFI of phospho-proteins relative to unstimulated controls (one representative experiment). (D) Relative phospho-protein levels (mean $\pm$ SEM, n=3). * $P < 0.0455$. (stim = stimulated, d + s = dasatinib + stimulated). (E) Dasatinib-induced reduction of pBtk and pITK basal levels, shown by flow cytometry analysis of CD20⁺ B cells and CD20⁻ T cells stained for pBtk/pItk. One representative experiment is shown (left) and quantification (right; mean $\pm$ SEM, n=5). ** $P < 0.0015$.

Figure 3. Dasatinib induces a more pronounced induction of apoptosis compared to ibrutinib in human peripheral B cells. (A) PBMCs were pretreated with or without ibrutinib (ibrut, 1 μ M) or dasatinib (das, 128 nM) for 1 h before stimulation with anti-IgM F(ab')₂ for 4 min, as described in 1). Shown is heat map of phospho-protein levels relative to unstimulated controls, one representative out of three independent experiments. (B) Relative phospho-protein levels (mean $\pm$ SEM, n=3). (C) CD19⁺ peripheral blood B cells were exposed to ibrutinib (ibrut) or dasatinib (das) for 5 days, exposed to PI and analyzed by flow cytometry (mean $\pm$ SEM, n=4). ** $P < 0.0024$. (D) CD19⁺ peripheral blood B cells were exposed to ibrutinib or dasatinib for 4 days, fixed and TUNEL stained and analyzed by flow cytometry where MFIs were measured. Shown is one representative experiment out of three independent experiments. (E) Quantification of the TUNEL staining presented in D (mean $\pm$ SEM, n=3). ** $P < 0.0017$.

Figure 4. Human precursor B cells from the pre-B stage are sensitive to dasatinib

exposure. CD19⁺ cells were isolated using M-450 anti-CD19 Dynabeads and CD19 DETACHaBEADs (Lifetechnologies, Oslo Norway). B cell precursors from BM aspirates were stained with anti-CD19, -CD34, -IgM, and CD10 and sorted into pro-B cells (CD19⁺/CD34⁺/IgM⁻), pre-B cells (CD19⁺/CD34⁻/IgM⁻) and immature B cells (CD19⁺/CD34⁻/IgM⁺), in addition to mature B cells (CD19⁺/CD10⁻) from the contaminated blood. (A) Gating strategies for sorting of B cell precursors from human BM aspirates. Pro-B cells (CD19⁺/CD34⁺/IgM⁻), pre-B cells (CD19⁺/CD34⁻/IgM⁻) and immature B cells (CD19⁺/CD34⁻/IgM⁺), in addition to mature B cells (CD19⁺/CD10⁻) from the blood, were sorted. A FACS Aria flow cytometer (BD) was used for sorting and data analysis was performed with Cytobank software (www.cytobank.org) or FlowJo (Tree Star, Ashland, OR). (B) The different cell populations (1×10^4 to 1×10^5 /well) were co-cultured separately with MS-5 stroma cells (8×10^3 /well) in X-VIVO15 medium (BioWhittaker, Switzerland) with IL-7 (50 ng/ml) and Flt-3 ligand (50 ng/ml) $\pm$ dasatinib (dasa, 128 nM) for 3 days. Cell viability was measured by exposure to PI followed by flow cytometric analysis. Shown is one representative out of three independent experiments; mean $\pm$ SEM, (n=3). * $P < 0.0253$, # $P < 0.0187$, (ctr = unstimulated control).

Figure 5. Dasatinib dosing of mice reduces B-lineage cells in the BM. Mice were dosed with vehicle or dasatinib for 4 weeks (n=5 and n=6 respectively), and the BM cells analyzed by flow cytometry. (A) Representative profiles from a vehicle and a dasatinib treated mouse stained with anti-B220 and anti-IgM antibodies. The profiles show dasatinib induces a marked reduction in the percentages of IgM⁻ and IgM⁺ cells within the B220⁺ population. (B) Cumulative flow cytometry data showing the significant loss of B lineage cells in the BM from dasatinib treated mice. (C) Representative profiles from a vehicle and a dasatinib treated

mouse stained with anti-Gr-1 and anti-CD11b antibodies showing the increase in the percentage of myeloid-lineage cells from the dasatinib treated mouse. (D) Cumulative flow cytometry data showing the significant increase in the percentage of myeloid lineage cells in the BM from dasatinib treated mice. (E) Representative profiles of B220⁺ IgM⁻ gated cells stained with anti-CD24 and anti-CD43 antibodies showing the significant loss Pre-B cells, i.e. cells expressing high levels of CD24 and low levels of CD43. The gates that define the Pre-B, Pro-B and PrePro-B populations are shown. (F) Cumulative flow cytometry data showing the significant decrease in the percentage of Pre-B cells in the BM from dasatinib treated mice, and the corresponding increase in the percentages of Pro-B and PrePro-B cells. (G) Analysis showing no effect of dasatinib on the percentages of lineage negative (Lin⁻); Lin⁻ c-Kit⁺ (LK); Lin⁻, Sca-1⁺, c-Kit⁺ (LSK); multi-potent progenitors (MPPs; defined as FLT3⁺ LSK cells), long-term hematopoietic stem cells (LT-HSCs, defined as CD150⁺ CD48⁻ LSK cells); Lin⁻ cells expressing low levels of Sca-1 and c-Kit (LSK^{lo}) and common lymphoid progenitors (CLPs, defined as IL-R⁺ FLT3⁺ LSK^{lo} cells). Percentages are of the indicated parent populations shown in parenthesis. (H) After 4 weeks of dosing mice were bled by cardiac puncture and their serum assayed for SCF levels by ELISA. The results show dasatinib promotes a significant induction of SCF. The results are expressed as mean $\pm$ SEM. ** P < 0.01, *** P < 0.001, **** P < 0.0001 using unpaired Student's t test.

Figure 6. Dasatinib targets immature B cells in the spleen. Mice were dosed with vehicle or dasatinib for 4 weeks (n=5 and n=6 respectively) and analyzed for (A) spleen weights. (B) Representative flow cytometry profiles of B220⁺ gated spleen cells analyzed for the expression of IgM and IgD. The profiles show that dasatinib markedly reduces the percentages of immature transitional (T1 and T2) B cells, but not mature follicular B cells. (C) Cumulative data showing significant losses of immature transitional B cells, and the

corresponding increase in the percentage of follicular B cells. (D) Dasatinib specifically deletes large immature T1 and T2 cells, but has no effect on small follicular B cells. Shown are representative forward light scatter (FSC) profiles of T1, T2 and follicular B cells from a vehicle and a dasatinib treated mouse. (E) Germinal center B cells are markedly reduced in dasatinib treated mice. Shown are representative flow cytometry profiles of B220⁺ gated cells stained with anti-GL7 and anti-CD95 antibodies, and the cumulative data for the percentages GL7⁺ and CD95⁺ germinal center cells. (F) The percentages of T cells and myeloid cells in the spleen are unaffected by dasatinib. The cumulative data is from flow cytometry analysis with anti-TCR and anti-CD11b antibodies. The results are expressed as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ using unpaired Student's t test.

Figure 7. Dasatinib causes a marked loss of thymocytes. Mice were dosed with vehicle or dasatinib for 4 weeks (n=3 in both groups). (A) Total thymocyte numbers and (B) numbers of double negative (DN), double positive (DP), and CD4 and CD8 single positive (SP) thymocytes are significantly reduced in dasatinib treated mice. (C) Representative CD4/CD8 flow cytometry profiles from a vehicle and a dasatinib treated mouse showing the percentages of each population. (D) Cumulative flow cytometry data showing the percentages each of the four thymocyte populations. The analysis showed that DN thymocytes, which are less reliant on SFK signaling, is the population least affected by dasatinib. The results are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using the unpaired Student's t test.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

- Dasatinib treatment in mice had pronounced negative effect on B- and T-lineage cells
- Dasatinib induced loss of human blood B cells and BM progenitors at the pre-B cell maturation stage
- The effects of dasatinib may have an impact for patients receiving long-term treatment

Supplemental Figure 1. Ibrutinib or dasatinib does not abrogate the CD40 signaling pathway in peripheral B cells. (A) PBMCs were incubated $\pm$ dasatinib (das; 128 nM) or ibrutinib (ibrut; 1 μ M) for 1 hour before stimulation with CD40L for 15 minutes. Fixed cells were bar coded with pacific blue and stained with anti-CD5/CD20 (for gating of CD20⁺ B cells) in addition to the specified phospho specific mAb and analyzed by flow cytometry. The heat map illustrates MFI of phospho-proteins relative to unstimulated controls for one representative result out of three independent experiments. (B) Quantification of the phosphorylations signals (MFI) presented in A. Shown is mean $\pm$ SEM (three different blood donors; n=3). (C) PBMCs were incubated $\pm$ dasatinib (dasa; 128 nM) for 1 hour before stimulation with CD40L for 15 minutes to 3 hour. Cells were treated and analyzed as described in A. The heat map illustrates MFI of phospho-proteins relative to unstimulated controls for two independent experiments representing two different blood donors.

Supplemental Figure 2. Dasatinib does not interfere with the IL-2 signaling pathway in peripheral T cells. PBMCs were incubated $\pm$ dasatinib (128 nM) for 1 h before stimulation with IL-2 for 15 min. Fixed cells were bar coded with pacific blue and stained with anti-CD5/CD20 (for gating of CD20⁺ B cells) in addition to the specified phospho specific mAb. The heat map illustrates MFI of phospho-proteins relative to unstimulated controls. Shown are two independent experiments (different blood donors).

Supplemental Figure 3. Gating strategies for hematopoietic stem cells and progenitors. Gates used to determine LSK cells and long-term hematopoietic stem cells (LT-HSCs) are shown in (A). Gating for and multipotent progenitors (MPPs) and common lymphoid progenitors (CLPs) are shown in (C). The single parameter c-Kit profiles in (B) reveal a slight reduction in c-Kit expression on LSK cells and LT-HSCs, and this shift is statistically significant when

quantified by mean fluorescence intensity. The results are expressed as mean $\pm$ SEM using the unpaired Student's *t* test. **** $P < 0.0001$. N=4 for vehicle dosed mice and N=6 for dasatinib dosed mice.

Supplemental Figure 4. Dasatinib induces the death of cultured mouse splenic B cells.

Splenic B cells from a C57BL/6 mouse were cultured for 3 days with 250 ng/ml of CD40L in the presence of DMSO or 128 nM dasatinib. Cell death was determined by propidium iodide staining (4 μ g/ml) and flow cytometry. Data is mean $\pm$ standard error from triplicate wells.

Supplemental Figure 5. Dasatinib dosing does not reduce the numbers of peripheral white

blood cells. Mice were bled from the tail vein before dosing and after 4 weeks of dosing with vehicle or dasatinib (n=5 and n=6 respectively), and blood was analyzed by Hemavet differential counting. (A) Total white blood cell (WBC) counts and the percentages of (B) lymphocytes and (C) neutrophils were unaffected by dasatinib. Flow cytometry analysis showed dasatinib did not alter the percentages of (D) CD19⁺, (E) TCR⁺ or (F) CD11b⁺ WBCs.

Supplementary Figure 1

Supplementary figure 2

Supplementary Figure 3

Supplementary Figure 4

Supplementary Fig. 5