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Dasatinib promotes the activation of quiescent hematopoietic stem cells in mice

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ABSTRACT

Dasatinib is an orally available broad-spectrum tyrosine kinase inhibitor that is widely used to treat chronic myeloid leukemia. It is also in clinical trials to treat other malignancies including solid tumors. Despite its wide use, little is known about its effects on normal hematopoietic stem and progenitor cells. Here we study wild-type mice dosed with dasatinib and find that it causes the transient induction of proliferation of quiescent hematopoietic stem cells (HSCs). This finding was unexpected given dasatinib's ability to inhibit c-Kit signaling and promote cell cycle arrest in many cell types. The transient induction of HSC proliferation in dasatinib-dosed mice coincided with a marked induction in the expression of Sca-1 and phospho-S6. Also evident at this time was a rapid but transient loss of lineage-committed hematopoietic progenitors that express high levels of c-Kit, and the induction of stem cell factor (SCF) in the serum. These findings suggest that activation of quiescent HSCs is part of a rapid rescue response that restores hematopoietic progenitors to pre-treatment levels. This restoration coincides with HSCs returning to quiescence, and the expression of Sca-1 and phospho-S6 reverting to pre-treatment levels, even though dasatinib dosing is maintained. These data suggest that equilibrium is reached between the opposing forces of dasatinib and hematopoietic growth factors. The transient induction of HSC proliferation provided a window of opportunity whereby these cells became sensitive to killing by the cytotoxic drug 5-fluorouracil.

INTRODUCTION

Dasatinib is a second-generation tyrosine kinase inhibitor (TKI) that is widely used for the treatment of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia. It is commonly used for treating CML patients who have developed resistance to earlier TKI regimens, although more recently, along with nilotinib, it is becoming a preferred first-line treatment choice [1-3]. It potently inhibits Bcr-Abl and Src-family kinases, and receptor tyrosine kinases c-Kit, PDGFR- α and - β , and Eph [4,5]. Dasatinib is also being investigated as a treatment for some B cell lymphomas, due to its inhibition of Lyn and Bruton's tyrosine kinase [6-9], as well as other malignancies including solid tumors [10].

Since the majority of studies have focused on dasatinib's effect on hematopoietic disorders there have been few studies addressing the impact of dasatinib on normal blood cells, especially *in vivo*. To address this we recently investigated the effects of dasatinib on hematopoiesis in both wild-type mice and mice with a knock-in mutation in the RING finger domain of c-Cbl [11,12], a mutation that promotes the development of a myeloproliferative disease (MPD) [13]. In these studies we found that although dasatinib did not provide an effective therapy for c-Cbl-driven MPD, it did markedly reduced immature B-lineage cells and thymocytes. Surprisingly however dasatinib did not affect the numbers of hematopoietic stem cells (HSC) or progenitors. This was unexpected given dasatinib's ability to inhibit c-Kit, and the *in vivo* requirement of these cells for c-Kit signaling [14-16]. We did however find that serum from dasatinib-dosed mice showed higher levels of stem cell factor (SCF), i.e. c-Kit ligand, compared to vehicle-treated mice after 4 weeks of dosing [11]. This finding raised the possibility that perturbations to HSCs or progenitors may occur at earlier times, and that these may have been rescued by an SCF response that compensated for the inhibitory effects of dasatinib. In this study we find that dasatinib induces the transient

activation of quiescent HSCs, a property that could potentially be utilized for activating quiescent leukemia stem cells that are resistant to cytotoxic drugs.

MATERIALS and METHODS

Mice

Male C57BL/6 and C57BL/6.CD45.1 mice 8-10 weeks old were purchased from the Animal Resource Centre (Canning Vale, Western Australia). All mouse experiments were performed in accordance with the regulations of the Animal Ethics Committee at the University of Western Australia (approval 100/1169).

Dasatinib, imatinib and 5-fluorouracil

Dasatinib was obtained from LC laboratories (Woburn, MA) and a 5x stock solution was prepared in 80 mM Citric Acid, pH 2.1, and diluted 1 in 5 with 80 mM Citrate Buffer pH 3.1 before dosing. Mice were dosed twice daily with 15 mg/kg of dasatinib or citrate buffer vehicle by oral gavage using 20G 1.5 inch feeding needles (Braintree, MA). Imatinib was obtained from LC laboratories (Woburn, MA) and stock solutions prepared in 50% sucrose (to lessen the bitter taste). Mice were dosed with 100 mg/kg imatinib or vehicle twice daily. 5-Fluorouracil (5-FU) was obtained from Sandoz (Pymont, NSW) as a 50 mg/ml solution and diluted in phosphate buffered saline for a single intraperitoneal injection of 150 mg/kg.

Analysis of bone marrow cells and blood serum

Bone marrow cells were prepared by flushing cells from two tibias and femurs of each mouse and were analyzed by flow cytometry (FACS Canto, BD Biosciences) using monoclonal antibodies and procedures described in Supplementary Materials and Methods. Data was collected using FACSDiva version 6 software (BD Biosciences) and analyzed using FlowJo 9.3.1 software (Tree Star, Inc., Ashland, OR).

Cardiac or tail vein blood was collected for serum and analyzed for levels of stem cell factor and FLT3 ligand (Quantikine Kit, R&D Systems Inc, Minneapolis, MN), or interferon- α (Platinum ELISA, eBioscience, San Diego, CA).

Statistical analyses

For days 1, 8 and 16 of dosing time-courses $n=3$ for vehicle, and 3, 4 and 5 respectively for dasatinib. For days 2 and 4 of dosing numerous experiments were performed. For analysis of the forward scatter of viable cells six experiments were performed with a total $n=19$ for both vehicle and dasatinib-dosed mice. For Ki-67, pS6 and pErk data $n=9$ from three experiments. For 7AAD DNA cell cycle analysis $n=10$ from three experiments for day 2 of dosing, and $n=17$ from five experiments for day 4. For BrdU analysis $n=6$ from two experiments for day 2, and $n=3$ from a single experiment for day 4. For imatinib experiments $n=4$ for vehicle and imatinib dosed mice on days 2 and 4. For 5-FU treatment two experiments were performed with $n=8$ for both vehicle and dasatinib-primed mice. To validate significance we used unpaired two-sided Student's t -tests for single experiments, or 2-way ANOVA where experiments were repeated (Prism 5, GraphPad Software). P values less than 0.05 were considered statistically significant. All statistical data are presented as means $\pm$ standard errors.

RESULTS

Dasatinib dosing induces the transient activation of quiescent long-term HSCs

From our previous studies of both wild-type C57BL/6 mice and c-Cbl RING finger mutant mice we observed that dasatinib did not reduce HSC numbers after 4 weeks of daily dosing [11,12]. To investigate this further we analyzed C57BL/6 mice over a 16-day time course of twice daily dosing of 15 mg/kg of dasatinib, or vehicle, to determine whether dynamic, yet transient, effects were evident at earlier time points. The study involved an analysis of cell size, Ki-67 expression

and cell cycle status of long-term (LT)-HSCs, short-term (ST)-HSCs, and multipotent progenitors (MPPs) to determine the *in vivo* effects of dasatinib on these cells. The flow cytometry gating strategies to identify these populations are shown in Supplementary Figure 1, and are based on published procedures [17,18]. Strikingly, after 2 and 4 days of dasatinib dosing we observed significant increases in cell size and the induction of Ki-67 expression within the LT-HSC population (Fig. 1A and B). By days 8 and 16 the population of large LT-HSCs had returned to vehicle-treated sizes, and consistent with this the proportion of Ki-67⁺ cells was also markedly reduced. This time course analysis therefore demonstrated that dasatinib induces a transient activation LT-HSCs. Additional cohorts of mice were dosed for 2 and 4 days to determine cell cycle DNA profiles (Fig. 1C). These studies confirmed that LT-HSCs from dasatinib-dosed mice were induced to cycle by exhibiting a significant increase in the percentage of cells in S, G2 and M phases of the cell cycle.

These analyses indicated that dasatinib promotes the proliferation of HSCs, however to determine whether HSCs continue to cycle more rapidly after 2 and 4 days of dasatinib dosing we injected mice with 5-bromodeoxyuridine (BrdU) 3 hours before harvesting bone marrow cells. These experiments involved the analysis of BrdU incorporation into the DNA of FLT3 negative LSK cells, rather than CD150⁺ CD48⁻ LSK cells. This modified approach was necessary because of technical difficulties associated with our inability to detect CD150⁺ cells that were stained prior to or following fixing and permeabilization. By excluding the highly proliferative FLT3 positive MPPs the LSK cells are more highly enriched for quiescent cells than the total LSK population. The analysis showed that mice dosed with dasatinib for 2 or 4 days exhibited a greater increase in the proportion of FLT3 negative LSK cells that incorporated BrdU compared to vehicle dosed mice (Fig. 1D). Thus, the BrdU incorporation data suggests that HSCs continue to cycle more actively after 2 or 4 days of dasatinib exposure.

A recent study revealed that c-Kit^{lo} cells within the LSK CD150⁺ population contain a higher proportion of Ki-67 negative cells that exhibit greater long-term reconstitution potential than c-Kit^{hi} cells within this population [19]. We therefore analyzed c-Kit^{lo} CD150⁺ CD48⁻ LSK cells for the parameters described above. Consistent with published data we found that c-Kit^{lo} cells exhibited a lower proportion of Ki-67⁺ cycling cells compared to the total c-Kit⁺ population within the CD150⁺ CD48⁻ LSK gate (compare vehicle-treated mice in Fig. 1B with vehicle-treated mice in Fig. 1F). Significantly, we observed that c-Kit^{lo} cells responded to dasatinib dosing through an induction in cell size, Ki-67 expression, and an increase in the proportion of cells in S, G2 and M phases of the cell cycle (Fig. 1E, F and G).

In contrast to the *in vivo* effects of dasatinib, we found that *in vitro* treatment with 128 nM of dasatinib in cultures of lineage negative bone marrow cells did not activate LT-HSCs, but rather dasatinib induced a reduction in the proportion of Ki-67⁺ cells (Supplementary Figure 2). This effect was most evident in cultures that did not contain hematopoietic growth factors (Supplementary Figure 2A and C). Not surprisingly, when growth factors were present, they induced a high level of proliferation in LT-HSCs, and as a consequence dasatinib was less effective in inhibiting their proliferation (Supplementary Figure 2B and D). In summary, these experiments indicate that the *in vivo* effects of dasatinib in transiently activating LT-HSCs are likely to be indirect.

Effects of dasatinib dosing on ST-HSCs, MPPs and LK cells

Similar but less profound effects were observed for ST-HSCs (CD150⁻ CD48⁻ LSK cells), with increases in cell size on days 2 and 4 of dasatinib dosing (Fig. 2A). Consistent with this, statistically significant increases in Ki-67 expression, and S, G2 and M phases of the cells cycle,

were observed on days 2 and 4 (Fig. 2B and C respectively). The effects of dasatinib on MPPs were also analyzed (Fig. 2D-F), and although an increase in large proliferative cells was evident it was not as marked as that seen for either LT- or ST-HSCs. This was not surprising given that MPPs are inherently more proliferative. Indeed dasatinib dosing causes a conversion of LT-HSCs towards a proliferative phenotype that more closely matches that of vehicle-treated MPPs, with both populations approximating 70-80% Ki-67⁺ cells. Interestingly dasatinib's effects on MPPs contrasts results we observed with the FLT3 inhibitor AC220, or quizartinib, which induced the transient quiescence of MPPs, before they were rescued by a robust FLT3 ligand-driven proliferative response [20]. This study involved the dosing of c-Cbl RING finger mutant mice, however we have observed a similar response in MPPs from wild-type mice (unpublished).

The effects of dasatinib on the proliferation of Lin⁻ Sca-1⁻ c-Kit⁺ (LK) cells were found to be minimal with no significant increase in cell size (Supplementary Figure 3A). A slight increase in the Ki-67 expression was evident at day 4, and small enhancement in the percentage of cells in S, G2 and M phases of the cell cycle on days 2 and 4 (Supplementary Figure 3B and C). LK cells, that contain committed myeloid and erythroid progenitors, are highly proliferative and therefore it was not surprising that dasatinib dosing did not markedly enhance this activity.

Short-term dasatinib dosing does not alter the repopulating ability of HSCs

To determine whether the transient activation of HSCs affects their ability to repopulate lethally irradiated recipient mice we dosed B6.CD45.1 mice twice daily with dasatinib or vehicle for 2 or 4 days before harvesting bone marrow. The bone marrow cells from the dosed mice were mixed equally with competitor bone marrow from an untreated B6.CD45.2 mouse, and a total of 10⁶ cells were injected into irradiated B6.CD45.1 recipients (Fig. 3A). Peripheral blood from the four groups was analyzed by flow cytometry at 10 and 21 weeks after transplantation to determine the

proportional contribution by each of the donor bone marrows. We found no significant difference between the contributions by untreated and treated donor bone marrows in repopulating either the myeloid or B lymphoid lineages (Fig. 3B). Thus HSCs from dasatinib-dosed mice do not appear to be functionally altered in their ability to establish and maintain hematopoiesis.

Dasatinib dosing results in a loss of LK cells expressing high levels of c-Kit

The above data does not distinguish whether the effects of dasatinib on HSC proliferative activity are direct or indirect. A possible explanation for an indirect effect is that the HSCs are responding to replenish bone marrow cells that are lost due to dasatinib-induced toxicity. To support this possibility we found a significant loss of bone marrow cells by days 2 and 4 of dasatinib dosing. Interestingly, a partial recovery of cell numbers occurred by day 8, and a recovery approaching vehicle-treated levels was evident by day 16 (Fig. 4A). Importantly the timing of the recovery in the bone marrow coincided with HSCs returning to a predominantly quiescent state (Fig. 1A and B). These findings provide support for an indirect effect on HSCs in that they are transiently responding to replenish bone marrow cellularity.

Further characterization of the bone marrow revealed that the loss of cells was most noticeable within the LK population (i.e. Lin⁻, c-Kit⁺, Sca-1⁻ cells) (Fig. 4B and C), and that this loss was most profound for cells expressing high levels of c-Kit (Fig. 4B, D and E). Interestingly, the number of c-Kit^{hi} LK cells had not fully recovered by day 16, which is in contrast to total LK cell numbers that returned to vehicle-treated levels by this time (compare day 16 in Fig. 4C and E). This suggests that c-Kit^{hi} LK cells could not be fully restored by compensatory responses that are likely to be triggered in mice dosed with dasatinib.

Since LK cells are enriched for committed progenitors we determined whether there was a specific progenitor population that was depleted by dasatinib. We examined cell-surface markers that define common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) after two days of dosing with dasatinib or vehicle. We found no significant differences among these populations suggesting that dasatinib was equally potent in deleting all three populations of progenitors (data not shown).

The loss of LK cells is in contrast to LT-HSCs where the proportion and numbers of these cells were increased after 2 and 4 days of dasatinib dosing (Supplementary Figure 4A and D). The analysis also revealed a small but significant reduction of ST-HSCs on these days, but an increase in MPPs (Supplementary Figure 4B, C, E and F). Consistent with these findings dasatinib did not promote any significant apoptotic effects on CD150⁺ or CD150⁻ LSK cells as assayed by Annexin V and 7-AAD staining (Supplementary Figure 5). Thus the LSK compartment appears to be protected against the acute loss of cells that is evident in the LK fraction following dasatinib treatment.

Dasatinib dosing causes a marked induction of Sca-1 expression

An additional effect of dasatinib on the phenotype of lineage negative cells was a marked induction of Sca-1 expression (Fig. 4E). The increase in Sca-1 expression can also be seen in the Sca-1 vs c-Kit flow cytometry profiles from mice dosed for 2 days with either vehicle or dasatinib (Fig. 4B). As with other phenotypic changes associated with dasatinib the effect was transient, peaking at days 2 and 4, and returning to vehicle-treated levels by days 8 and 16. An increase in Sca-1 expression, albeit more profound, has also been observed in mice where type 1 interferons are induced following treatment with poly I:C [21,22]. In one of these studies the increase in Sca-1 expression was clearly evident in c-Kit⁺ CD48⁻ CD150⁺ gated cells [22], and this effect was also

seen in mice dosed for 2 and 4 days with dasatinib (Fig. 4F). These cells encompass LT-HSCs examined in Fig. 1, and therefore the aberrant induction of Sca-1 expression provides further evidence that these cells are profoundly affected by dasatinib dosing. Whether the increase in Sca-1 has a functional role is not known.

Dasatinib induces stem cell factor production and promotes the induction of phospho-S6

A possible contributor to the proliferative activity of HSCs from mice dosed with dasatinib is the induction of SCF. To examine this possibility sera from mice dosed with dasatinib or vehicle for 1 to 16 days were analyzed by ELISA. We found that SCF was increased from day 1 of dasatinib dosing, and, unlike the transient cellular effects, remained elevated at day 16 (Fig. 5A). We also analyzed sera from B6 mice that had been dosed daily with dasatinib for 4 weeks and observed a similar sustained level of SCF (Fig. 5A). Analysis of FLT3 ligand found that this hematopoietic growth factor was also elevated in response to dasatinib (Fig. 5B), albeit significantly lower than the levels induced in mice following dosing with the FLT3 inhibitor quizartinib [20]. Since HSC proliferation and Sca-1 induction occurs in mice exposed to type 1 interferons, we also examined interferon α levels, however no evidence of induction by dasatinib was found (data not shown). Thus it appears that the similar phenotypic effects caused by dasatinib and poly I:C may involve distinct mechanisms.

To further investigate the activation status of HSCs from dasatinib dosed mice we analyzed signaling by the PI 3-kinase and Ras pathways by staining LSK cells with pS6 and pErk antibodies respectively. The flow cytometry analysis showed a transient induction of pS6 on days 2 and 4, but no evidence of pErk induction (Fig. 5C and D). A significant level of activation of pS6 was also evident in LK cells on days 2 and 4 (Fig. 5F). Thus it appears that LK cells, that were not lost

by dasatinib dosing, were capable of responding to growth factors produced at this time. An increase in pS6 was also observed in Lin⁻ c-Kit⁻ bone marrow cells on day 4 of dasatinib dosing (Fig. 5G). These cells express neither c-Kit nor FLT3, suggesting that activation of these cells is likely to be mediated by growth factors other than SCF and FLT3 ligand.

Imatinib causes a loss of c-Kit⁺ cells but does not induce an HSC proliferative response

Since imatinib is another well-characterized and widely used c-Kit inhibitor we examined its effects on c-Kit⁺ cells and HSC proliferation. Mice were dosed with 100 mg/kg of imatinib twice daily for 2 or 4 days before analysis. This dose approximates 500 mg twice daily for humans, which is slightly higher than the 400 mg twice-daily dose recommended for patients in accelerated or blast crisis phases of CML [23]. We found that imatinib caused a decrease in LK cells at days 2 and 4 (Fig. 6A and B), and this effect was more pronounced when focused on the loss of c-Kit^{hi} cells (Fig. 6C and D). These findings were similar to, but not as profound, as the depletion observed in dasatinib-dosed mice at days 2 and 4 (compare with Fig. 4D and E). Most telling however, imatinib did not markedly enhance the proliferation status of LT-HSCs (Fig. 6E, F and G), or any other population within the LSK gate (data not shown), as measured by FSC, Ki-67 expression and DNA content. Another significant distinction between the effects of dasatinib and imatinib was the minimal induction of Sca-1 in lineage negative cells on day 2 of imatinib dosing, and the absence of this induction on day 4 (Fig. 6H and I). This was in marked contrast to dasatinib-dosed mice (Fig. 4F). In addition, no detectable induction of SCF or pS6 was observed in LSK cells from imatinib-dosed mice (data not shown). Thus the loss of c-Kit⁺ cells in imatinib-dosed mice does not appear to be sufficiently robust to induce a detectable compensatory proliferative response within the HSC population. This data raises the possibility that additional dasatinib targets, such as Src family kinases, may be playing a role in promoting the responses that induce the HSC proliferative response.

Dasatinib priming increases the loss of HSCs following 5-fluorouracil treatment

The induction of proliferation in quiescent HSCs induced by dasatinib dosing suggests that these cells may become more susceptible to treatment with cytotoxic compounds that target cycling cells. This possibility was tested by dosing mice with vehicle or dasatinib (15 mg/kg twice daily) for 2 days, followed by an i.p. injection of 5-FU (150 mg/kg) one day later (Fig. 7A). Bone marrow cells were analyzed by flow cytometry for HSC markers 48 hours after 5-FU treatment. The results showed there was a significant loss of total bone marrow cells in the dasatinib + 5-FU treated mice compared to vehicle + 5-FU mice (Fig. 7B), and that this cell loss was more evident in LSK cells compared to LK cells (Fig 7C). Within the LSK population dasatinib promoted a significant reduction in the numbers of LT-HSCs (both c-Kit^{hi} and c-Kit^{lo}) and ST-HSCs, whereas the loss of MPPs was not significant (Fig. 7D). These findings indicate dasatinib can increase the sensitivity of HSCs to 5-FU-induced death, a population that normally contains a high proportion of quiescent, and therefore chemotherapy resistant, cells. Thus dasatinib priming has potential as an approach for enhancing the elimination of quiescent leukemia stem cells by anti-proliferative cytotoxic drugs.

DISCUSSION

The key finding from this study is that dasatinib promotes the transient activation of quiescent HSCs. This finding was unexpected given that dasatinib is an inhibitor of c-Kit signaling and has the ability to arrest the proliferative activity of many cell types [24-26]. However, these studies investigating dasatinib's ability to promote cell cycle arrest were carried out *in vitro*, highlighting the importance of studying animal models to reveal more biologically relevant outcomes.

At this point we do not know whether dasatinib's ability to promote HSC proliferation is an indirect or direct effect, or a combination of both. A possible mechanism is that the HSC activation is in response to the loss of proliferative lineage-committed progenitors that express high levels of c-Kit. These LK cells appear to be very sensitive to the cytotoxic effects of dasatinib, which is in contrast to MPPs, which, although highly proliferative, do not have an equivalent c-Kit^{hi} population. Thus dasatinib's ability to delete hematopoietic progenitors appears to be dependent on a high level of c-Kit expression.

The induction of SCF supports the hypothesis of a feedback rescue response that activates HSCs to replenish the loss of LK cells. Indeed by day 16 of dosing the LK population shows a recovery, and the HSC proliferative response is no longer detectable. Importantly, the SCF response is maintained over the period of dosing suggesting that equilibrium is achieved between the opposing forces of dasatinib and SCF. In contrast, dosing mice with imatinib did not induce an HSC proliferative response, even though it did promote the loss of c-Kit^{hi} LK cells, albeit less profound than the depletion mediated by dasatinib. This raises the possibility that there is a threshold for the requirement of an HSC response, whereby a less severe cytotoxic effect is inadequate to induce HSC activation. The absence of an increase in SCF levels in imatinib-dosed mice supports this possibility.

The only other studies to have revealed similar effects on HSC proliferation, and induce a high level of Sca-1 expression, are those investigating mice treated with poly I:C which induces the production of interferon α [21,22]. Similarly, a study treating mice with an MPL neutralizing antibody showed that it increased the frequency of proliferative HSCs [27]. Interestingly, this study also found that treatment with an anti-c-Kit neutralizing antibody did not activate the proliferative activity of HSCs. One of the poly I:C studies provided data that supports a model

whereby interferon α does not actively trigger the proliferation of HSCs *in vivo*, but instead causes HSC proliferation by transiently reducing the expression and activity of many genes that enforce quiescence [21]. It is therefore possible that dasatinib's ability to inhibit Src family kinases could be suppressing multiple HSC quiescence-enforcing mechanisms, rather than activating the cell cycle machinery by growth factor stimulation. This possibility should be investigated in future studies given the inability of imatinib, which does not inhibit Src family kinases, to promote HSC proliferation.

As mentioned above the effects we observed on HSCs were unexpected, and at present we do not have an explanation why the inhibitory effects of dasatinib appear unable to block the transient proliferative activity of HSCs. The challenge for an explanation is also confounded by the assumption that dasatinib's inhibitory effects would act on these cells before a growth factor response could be triggered. Thus, more investigation into the mechanism of this early response is required.

This study also raises questions about whether the findings can contribute towards a better understanding of how to more effectively combine dasatinib with cytotoxic drugs. Determining how to optimize the administration of chemotherapy with tyrosine kinase inhibitors is an important area of investigation, and a recent study revealed that dasatinib in combination with cytarabine and doxorubicin enhanced chemotherapy-induced targeting of primary murine acute myeloid leukemia (AML) stem cells [28]. This study provided support for the importance of clinical trials investigating whether the addition of dasatinib to chemotherapeutic regimens can enhance the targeting of AML leukemia stem cells [29,30]. From our data we propose that dasatinib priming before chemotherapy should be investigated as an approach that could optimize this combinational therapy.

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Figure Legends

Figure 1. Dasatinib induces the transient activation of quiescent LT-HSCs

C57BL/6 mice were dosed twice daily with either vehicle or dasatinib for 1, 2, 4, 8 or 16 days before analysis of CD150⁺ CD48⁻ LSK cells (i.e. LT-HSCs) by flow cytometry. (A) Representative forward light scatter (FSC) histograms, and cumulative data showing mean FSC of dasatinib and vehicle dosed mice in order to compare cell size at each time point. (B) Representative profiles of Ki-67 expression, and cumulative data showing the percentage of Ki-67⁺ LT-HSCs from vehicle and dasatinib dosed mice. The Ki-67⁺ gate was determined from the leading edge of the isotype control antibody. (C) Representative DNA histograms of 7AAD staining with gates showing percentage of cells in S, G2 or M phase of cell cycle, and cumulative data showing the percentage of cells in S, G2 or M phase after 2 and 4 days of dosing with vehicle or dasatinib. (D) Representative BrdU and 7AAD profiles gated to display BrdU positive cells after 3 hours of BrdU exposure. Bar graph shows percentage of BrdU positive cells after 2 or 4 days of dosing. Due to antibody panel design the population displayed is FLT3^{neg} LSK cells, which is enriched for ST and LT-HSCs. (E) Mean FSC, (F) percentage of Ki-67⁺ cells and (G) percentage of cells in S, G2 or M phase of the cell cycle for c-Kit^{lo} LT-HSCs (which are further enriched for quiescent cells) from mice dosed for 1, 2, 4, 8 or 16 days. The gating strategies for identifying the cell populations are shown in supplementary Figure 1. Results are expressed as means $\pm$ standard errors. * $P < 0.05$, **** $P < 0.0001$ using the unpaired Student's *t* test or 2-way ANOVA.

Figure 2. Dasatinib induces the transient activation of ST-HSCs and MPPs

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, 4, 8 or 16 days before analysis by flow cytometry. Results are for CD150⁻ CD48⁻ LSK cells (i.e. ST-HSCs) and CD150⁻ CD48⁺ LSK cells (MPPs) respectively. (A and D) Representative FSC histograms showing dasatinib and vehicle treated samples to compare cell size at each time point, and cumulative data of mean FSC. (B and E) Representative profiles of Ki-67 expression, and cumulative data showing the percentage of cells that are Ki-67⁺. The Ki-67⁺ gate is shown, and was determined from the leading edge of the isotype control antibody. (C and F) Representative 7AAD histograms showing

DNA staining at days 2 and 4, and graphed to show the mean percentage of cells that are in S, G2 or M phase of cell cycle, as gated in the histograms. The gating strategy for ST-HSCs and MPPs is shown in Supplementary Figure 1. Results are expressed as means $\pm$ standard errors. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using 2-way ANOVA.

Figure 3. Short-term dasatinib dosing does not alter the repopulating ability of HSCs

(A) B6.CD45.1 mice were dosed twice daily with vehicle or dasatinib for 2 or 4 days before bone marrow cells were harvested for transplantation. Bone marrow cells from the treated mice were mixed equally with bone marrow cells from an untreated C57BL/6 (CD45.2) mouse and injected into lethally irradiated B6.CD45.1 recipient mice. Peripheral blood was taken at 10 and 21 weeks after transplantation and was analyzed by flow cytometry to determine the proportional contributions from dosed (CD45.1) and untreated (CD45.2) donors. (B) The data shows the percentage of CD45.1⁺ cells within the total white blood cell (WBC) population, and the CD19⁺ and CD11b⁺ populations, for mice repopulated with day 2 or day 4 treated donor bone marrow + untreated bone marrow. The analysis was carried out on 5 transplanted mice in each group.

Figure 4. Dasatinib dosing results in a loss of LK cells expressing high levels of c-Kit

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, 4, 8 or 16 days before analysis by flow cytometry. (A) Total bone marrow counts from 2 femurs and 2 tibias. (B) Representative Sca-1 and c-Kit flow cytometry profiles of lineage negative cells showing the gate and percentage of Sca-1 negative c-Kit positive (LK) cells for 2 days of vehicle or dasatinib treatment, and (C) the cumulative data of the total number of LK cells at all time points. (D) Representative c-Kit histograms overlaying dasatinib and vehicle treated samples displaying gates for c-Kit^{hi} cells, and (E) cumulative data showing the number of c-Kit^{hi} LK cells. (F) Representative Sca-1 histograms of lineage negative cells, and graph of percentages of gated Sca-1 positive cells. (G) Representative Sca-1 histograms of lineage negative, c-Kit⁺ CD48⁻ CD150⁺ cells, and graph of percentages of gated Sca-1 positive cells. Results are expressed as means $\pm$ standard errors. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ using the unpaired Student's *t* test or 2-way ANOVA.

Figure 5. Growth factor induction and signaling responses to dasatinib dosing

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, 4, 8 or 16 days and analysed by ELISA and flow cytometry. Sera from mice dosed twice daily over a 16-day time-

course or for 4 weeks were analyzed for (A) SCF levels and (B) FLT3 ligand levels by ELISA. Representative flow cytometry profiles comparing LSK populations from vehicle and dasatinib samples for (C) phospho-S6 and (D) phospho-ERK expression. Fold changes in mean fluorescence intensity of phospho-S6 expression in dasatinib-dosed mice compared to vehicle-dosed mice for (E) LSK cells, (F) LK cells and (G) lineage negative, c-Kit negative cells. The gating strategy for the cell populations is shown in Supplemental Figure 1. Results are expressed as means $\pm$ standard errors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using the unpaired Student's t test or 2-way ANOVA.

Figure 6. Imatinib causes the loss of c-Kit⁺ cells but does not induce an HSC proliferative response

C57BL/6 mice were dosed with vehicle or imatinib (100 mg/kg twice daily) for 2 or 4 days and bone marrow cells were analyzed by flow cytometry. (A) Representative flow cytometry profiles of lineage negative cells showing Sca-1 and c-Kit expression. The gate shows the percentage of Sca-1 negative c-Kit positive (LK) cells for 2 days of vehicle or imatinib treatment. (B) Cumulative data of the total number of LK cells for 2 and 4 days of dosing. (C) Representative c-Kit histograms overlaying imatinib and vehicle treated samples, displaying gates for c-Kit^{hi} cells, and (D) cumulative data showing number of c-Kit^{hi} LK cells. Cumulative data for (E) mean FSC, (F) percentage Ki-67⁺ cells and (G) percentage in cell cycle as measured by 7AAD DNA staining for LT-HSCs. (H) Representative Sca-1 histograms of lineage negative cells, and (I) percentages of Sca-1 positive cells. Results are expressed as means $\pm$ standard errors. ** $P < 0.01$, **** $P < 0.0001$ using the unpaired Student's t test.

Figure 7. Dasatinib priming enhances the loss of HSCs following 5-fluorouracil treatment.

(A) C57BL/6 mice were dosed with vehicle or dasatinib (15 mg/kg twice daily) for two days followed by an i.p. injection of 5-FU (50 mg/kg) on day 3. Bone marrow cells were analyzed 48 hours later. (B) Total bone marrow cell numbers from mice 48 hours after 5-FU treatment. (C) Cumulative data from vehicle + 5-FU and dasatinib + 5-FU treated mice showing numbers of LSK and LK cells. (D) Cumulative data from vehicle + 5-FU and dasatinib + 5-FU treated mice showing numbers of LT-HSCs, c-Kit^{lo} LT-HSCs, ST-HSCs and MPPs. The results are expressed as means $\pm$ standard errors. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using 2-way ANOVA.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

HIGHLIGHTS

- Dasatinib dosing of mice transiently activates quiescent hematopoietic stem cells.
- This activation corresponds with an induction of stem cell factor.
- 5-Fluorouracil killing of hematopoietic stem cells is enhanced following dasatinib dosing.

Supplementary Materials and Methods**Flow cytometry antibodies and procedures**

All antibodies are from BD Biosciences, except where noted otherwise. Biotin-labeled anti-CD3, CD11b, Gr-1, B220, TER119, anti-CD48-FITC or PE, CD150-PE-Cy7 (Biolegend), c-Kit-APC or PE, Sca-1-PE-Cy7 (eBioscience), PE or FITC, FLT3-PE (eBioscience). Cells stained with biotinylated antibodies were visualized with streptavidin conjugated with APC-Cy7 or PCP-Cy5.5 (BD Biosciences). Long-term hematopoietic stem cells (LT-HSCs) were identified as CD150⁺, CD48⁻ LSK cells, short-term (ST)-HSCs as CD150⁻, CD48⁻ LSK cells, multipotent progenitors (MPPs) as FLT3⁺ LSK cells or CD150⁻, CD48⁺ LSK cells [17,18]. Blood cells from competitive transplanted recipient mice were analyzed with CD45.1-FITC and CD45.2-APC antibodies and CD19-PE and CD11b-PE-Cy7 lineage antibodies. Ki-67 expression was determined by examining bone marrow cells fixed with Cytofix/Cytoperm (BD Biosciences), permeabilized with Perm/Wash buffer (BD Biosciences) and stained with either anti-Ki-67-FITC antibodies or FITC-labeled isotype control antibodies (BD Biosciences). Cell cycle analysis was performed by permeabilizing bone marrow cells with phosphate buffered saline containing 0.1% saponin (Sigma-Aldrich), 5% newborn calf serum and 250 mg/ml sodium azide. DNA was stained with 7-AAD (BD Biosciences) to a final concentration of 2.5 µg/ml. Cells were left at 4°C for two hours and at room temperature for 30 min before analysis by flow cytometry. For assaying in vivo proliferation of bone marrow cells, mice were injected intraperitoneally with 2 mg BrdU 3 hours before bone marrow harvest. Bone marrow cells were fixed, permeabilized and stained with BrdU antibodies according to the manufacturer's directions (BD Pharmingen BrdU Flow Kit). Cells were stained with lineage antibodies prior to fixing and permeabilization, and with antibodies to stem cell markers after. Apoptotic effects of dasatinib on HSCs were determined using an Annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's directions. Expression of phospho(p)-S6 ribosomal protein and pErk was determined by flow cytometry on cells fixed with Cytofix™ (BD Biosciences), permeabilized

with ice-cold methanol and stained with AF-647 conjugated pS6 and pErk antibodies (Cell Signaling Technology, Danvers, MA). Bone marrow cells examined for Ki-67 expression, pS6, pErk and cell cycle status were stained with fluorescence-labeled antibodies (to allow gating on HSC populations) before they were fixed or permeabilized, except for c-Kit staining, which was performed last.

In vitro treatment of HSCs and hematopoietic progenitors with dasatinib

Lineage negative bone marrow cells were purified from C57BL/6 mice using a Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set (BD Biosciences). The cells were cultured at 8×10^5 /ml in X-VIVO serum-free media (Lonza, Walkersville MD) in low attachment 30 mm culture dishes (Corning Costar), without growth factors or with a cocktail of SCF (50 ng/ml), IL-3 (6 ng/ml), IL-6 (10 ng/ml) and thrombopoietin (TPO) (50 ng/ml) (PeproTech, Rocky Hill, NJ). The cultures were exposed to 128 nM dasatinib or vehicle (DSMO) for 1 or 2 days before analysis by flow cytometry for Ki-67 expression of LT-HSCs. This dose was chosen because it is equivalent to the maximum serum concentration of dasatinib observed in CML patients taking FDA-approved doses (Dasatinib-Investigator Brochure, Bristol-Myers Squibb Pharmaceutical Research Institute, 15 October 2006 version).

Competitive bone marrow transplantation

C57BL/6.CD45.1 mice were lethally irradiated (2x 5.5 Gy) and repopulated by tail vein injection with donor bone marrow cells. The donor bone marrow cells were isolated from mice dosed twice daily with vehicle or dasatinib (15 mg/kg) for 2 or 4 days, and were mixed equally with competitor bone marrow from an untreated B6.CD45.2 mouse. A total of 10^6 cells were injected into irradiated B6.CD45.1 recipients. Peripheral blood was analyzed by flow cytometry at 10 and 21 weeks after transplantation to determine the proportional contribution by each of the donor bone marrows.

A **B** **C**

Supplementary Figure 1. Gating strategies for flow cytometry data

(A) Identifying Lin⁻ cells (1st panel), LSK cells and LK cells (2nd panel), LT-HSCs, ST-HSCs and MPPs (3rd panel), and c-Kit^{lo} LT-HSCs (4th Panel). Cells were stained with lineage markers, and antibodies for c-Kit, Sca-1, CD48 and CD150 then identified as shown. The lower panels show how the cell populations were examined for FSC (cell size, mean), Ki-67 (cell cycle) and 7AAD (DNA staining, gated to include S, G2 and M phase cells). (B) Identifying FLT3^{neg} LSK cells, and staining for 7AAD and BrdU to identify S phase cells. (C) Gating strategy for pS6 and pERK expression, measured on LSK, LK and Lineage^{neg} c-Kit^{neg} populations.

Supplementary Figure 2. Dasatinib does not induce the proliferation of LT-HCSs in vitro.

Lineage negative bone marrow cells were cultured for one or two days in the presence of 128 nM dasatinib or DMSO vehicle. The cells were cultured without growth factors (A and C), or with growth factors SCF, IL-3, IL-6 and TPO (B and D). The representative Ki-67 flow cytometry profiles are of gated CD150⁺ CD48⁻ LSK cells. The Ki-67 analysis of these cells showed that dasatinib significantly reduced the number of cycling cells when no growth factors were present (A and C), and caused a small reduction in cycling cells in the presence of growth factors (B and D). The data is from three experiments. * $P < 0.05$ and *** $P < 0.001$ using the unpaired Student's *t* test.

A

B

C

Supplementary Figure 3. Dasatinib induced proliferation is less marked in LK cells

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, 4, 8 or 16 days before analysis of Lin⁻, Sca-1⁻, c-Kit⁺ (LK) cells by flow cytometry. (A) Representative FSC histograms, overlaying dasatinib and vehicle treated samples to compare cell size at each time point and cumulative data showing mean FSC. (B) Representative profiles of Ki-67 expression, showing the Ki-67⁺ gate i.e proliferative cells and bar graphs showing the percentage of the cells that are Ki-67⁺. (C) Representative 7AAD histograms showing DNA staining at days 2 and 4 and cumulative data showing the percentage of cells that are in S, G2 or M phase of cell cycle. The gating strategy for the LK population is shown in Supplemental Figure 1A. n=3-5 for days 1, 8 and 16, n=19 from 6 repeats for days 2 and 4. Results are expressed as means $\pm$ standard errors. *P<0.05, **P < 0.01 using the unpaired Student's t test or 2-way Anova.

Supplementary Figure 4. Proportions and numbers of HSC and MPP populations.

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, 4, 8 or 16 days before analysis of Lin⁻, Sca-1⁺, c-Kit⁺ (LSK) cells by flow cytometry. Data is graphed as a percentage of bone marrow cells for (A) LT-HSCs, (B) ST-HSCs and (C) MPPs, and as total numbers of cells for the same populations respectively (D, E, F). The gating strategy for the populations is shown in Supplementary Figure 1A. n=3-5 for days 1, 8 and 16, n=19 from 6 repeats for days 2 and 4. Results are expressed as means $\pm$ standard errors. *P<0.05, ***P < 0.001, ****P<0.0001 using the unpaired Student's t test or 2-way ANOVA.

Supplementary Figure 5. Dasatinib does not induce significant apoptotic effects in Hematopoietic Stem and Progenitor Cells.

C57BL/6 mice were dosed twice daily with vehicle or dasatinib over 1, 2, or 4 days before analysis of Lin⁻, Sca-1⁺, c-Kit⁺ (LSK) cells by flow cytometry. CD150⁺ LSKs are enriched for LT-HSCs and CD150⁻ LSKs are enriched for Multipotent Progenitors. (A) Bar graphs of the percentage of cells that are Annexin⁺ 7-AAD⁻ (top row) or Annexin⁺ 7-AAD⁺ (bottom row). (B) Representative flow cytometry profiles from mice dosed for one day, gated for Annexin V and 7-AAD expression and showing percentages of cells within the gates. n=3 vehicle, and n=4 dasatinib and *P<0.05, **P < 0.01, using the unpaired Student's t test or 2-way ANOVA. Overall, the data shows that dasatinib dosing does not promote the death of LSK cells. The most profound effect we observed was a decrease in Annexin V⁺ 7-AAD⁺ CD150⁺ LSK cells from dasatinib dosed mice at day one. We also observed a slight but significant increase in Annexin V⁺ 7-AAD⁻ CD150⁻ LSK cells from dasatinib dosed on day two.