

## Effects of imatinib on bone marrow engraftment in syngeneic mice

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**Chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemias arise from the genetic reciprocal translocation t(9;22), forming the BCR-ABL fusion gene. These lead to the expression of the constitutively active tyrosine kinase BCR-ABL, which is the causative oncogene for these leukemias. Allogeneic bone marrow transplantation (BMT) or stem cell transplantation (SCT) is currently considered the only curative treatment for chronic myeloid leukemia (CML). Recently, the selective tyrosine kinase inhibitor imatinib mesylate (Glivec, formerly STI-571) has been shown to induce durable hematologic and major cytogenetic responses in a high percentage of patients with chronic phase CML. In patients with advanced disease remissions are transient and most patients relapse despite continued imatinib treatment. Some of these patients go on to receive allogeneic BMT or SCT, during which administration of imatinib is usually discontinued as it is believed to interfere with bone marrow engraftment. In this study, we examined the effect of imatinib on hematopoietic engraftment in a syngeneic mouse model. We found that imatinib has no significant influence on hematopoietic recovery in lethally irradiated mice *in vivo*. Thus, our results suggest that continued administration of imatinib in the course of BMT or SCT may be a feasible therapeutic regimen.**

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### Introduction

Imatinib is a competitive inhibitor at the ATP binding site of BCR-ABL, which plays a central role in the pathophysiology of Ph<sup>+</sup> malignancies.<sup>1</sup> Durable hematologic (98%) and major cytogenetic responses (31%) were observed in first clinical trials in a high percentage of patients in chronic phase CML treated with imatinib.<sup>2,3</sup> In contrast, imatinib-induced hematologic responses in advanced phase CML and Ph<sup>+</sup> ALL were not durable in most cases.<sup>4,5</sup> While imatinib is still at an early stage of clinical development and standard protocols for the most beneficial treatment have yet to be determined, allogeneic SCT remains the only proven curative therapy for CML.<sup>6</sup> Following the promising advances and the growing experience with imatinib, the results of allogeneic SCT for patients with CML or Ph<sup>+</sup> ALL might be improved by the use of imatinib. A combination regimen could possibly help to reduce relapse rates ranging between 5 and 35% among allografted recipients,<sup>6</sup> as well as to improve the outcome in autologous SCT.<sup>7</sup> The use of imatinib with high-dose chemotherapy and stem cell support might also prevent resistance against imatinib itself, which frequently develops in advanced Ph<sup>+</sup> leukemias.<sup>8–12</sup>

Indeed, recently published data indicate that imatinib is

able to improve the outcome in patients relapsing after autologous or allogeneic SCT.<sup>13–15</sup> Here, imatinib treatment was initiated after SCT and only mild to moderate adverse events were reported. So far, there have been no reports about the concomitant use of imatinib in the transplantation setting.

The major critical point in combining imatinib with BMT or SCT is the possible influence of imatinib on bone marrow engraftment. Imatinib is presumed to interfere with normal cellular function, since in addition to the inhibition of BCR-ABL, imatinib shows activity against other tyrosine kinases such as platelet-derived growth factor receptor and c-kit (CD117).<sup>16,17</sup> In particular, c-kit has been shown to play a crucial role in the regulation of early hematopoiesis.<sup>18–20</sup> Thus, imatinib administration during BMT/SCT is discontinued in the few clinical studies published to date, as administration of the tyrosine kinase inhibitor is believed to delay hematopoietic reconstitution.<sup>21,22</sup>

Using a syngeneic mouse transplantation model we addressed the question whether imatinib has a negative effect on bone marrow engraftment causing delayed hematopoietic reconstitution after BMT in mice.

### Materials and methods

#### Bone marrow harvest

Male donor Balb/C-mice were primed with 3 mg 5-fluorouracil (5-FU) 5 days prior to bone marrow harvest. Bone marrow cells flushed from the tibia and femur were counted, suspended in Hanks' balanced salt solution (HBSS) and used directly to either reconstitute lethally irradiated syngeneic female recipients or for *in vitro* assays.

#### Sorting of HSCs and clonal colony assay

Flow cytometric cell sorting (MoFlo cytometer, Cytomation, CO, USA) was performed after immunostaining of approximately  $1 \times 10^6$  mononuclear bone marrow cells (BMC) using FITC-conjugated Sca1 (Ly-6A/E) antibody and PE-labeled antibody against c-kit (CD117) (BD-Pharmingen, Heidelberg, Germany). Sorting gates were set to obtain Sca1<sup>+</sup> and Sca1<sup>-</sup> cells within the c-kit<sup>+</sup> population.

Freshly separated Sca1<sup>+</sup>/c-kit<sup>+</sup> and Sca1<sup>-</sup>/c-kit<sup>+</sup> HSCs were added to 1.2 ml methylcellulose medium (Methocult GF M3434; Stem Cell Technologies, Vancouver, Canada) and plated at a density of 400 cells per plate into 35 mm<sup>2</sup> tissue culture dishes in duplicate and incubated at 37°C, 5% CO<sub>2</sub>. After 9 days of culture, colonies consisting of more than eight cells were scored by standard morphological criteria using an inverted microscope.

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## Imatinib

Imatinib (Imatinib mesylate, formerly STI-571) was provided by Novartis Pharma, Basel, Switzerland. For *in vitro* experiments, stock solutions were prepared at 10 mM in distilled water. Preparations for administration to animals were made twice a day at the concentration of 5 mg/ml.

### Bone marrow transplantation

All animals were kept in a special caging system (Thoren, PA, USA) providing a specific pathogen-free environment and received acidified water and food *ad libitum*. All experiments using animals were reviewed and approved by the university supervisory animal care committee.

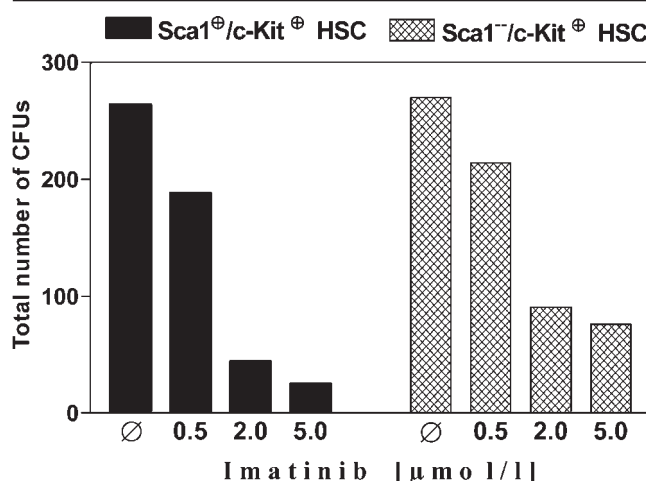
After lethal irradiation (800 rad total dose) recipient female Balb/C-mice 16 to 20 weeks of age (Harlan, Indianapolis, IN, USA) were transplanted with native mononuclear bone marrow cells (BMC) via tail vein injection. Seven days prior to BMT mice were started on imatinib or placebo, respectively, in order to ensure adequate therapeutic levels of imatinib during BMT. Oral administration of imatinib (at 25 mg/kg twice daily) was performed in a volume of 100  $\mu$ l sterile water by gavage. After BMT mice were monitored for reconstitution by obtaining blood counts at least every 2 days (Vet abc blood counter; Scil, Viernheim, Germany). After complete hematopoietic reconstitution, engraftment was assessed by secondary transplantation and by flow cytometry analyzing the Sca1 (Ly-6A/E), c-kit (CD117) and Thy1.2 (CD90) surface marker expression of freshly harvested bone marrow cells. Furthermore, we confirmed successful long-term engraftment by secondary transplantation of  $1 \times 10^6$  BMCs.

## Results

### Inhibitory effect of imatinib on c-kit<sup>+</sup> colony-forming cells *in vitro*

Previously published results showed an inhibitory effect of imatinib on normal hematopoiesis *in vitro*.<sup>17,23</sup> Imatinib is known to possess activity against the platelet-derived growth factor receptor and c-kit (CD117), which is critical in the survival and development of progenitor cells. Therefore, we tried to delineate the influence of imatinib on progenitor cells and long-term reconstitution *in vivo*.

First, we determined the inhibitory effect of imatinib on hematopoietic progenitor cell growth *in vitro* using clonal colony assays. We analyzed two separate murine populations with *in vivo* repopulation capacity both expressing the surface marker c-kit.<sup>24,25</sup> Bone marrow cells were fractionated by flow cytometric cell sorting into more primitive Sca1<sup>+</sup>/c-kit<sup>+</sup> stem cells and Sca1<sup>-</sup>/c-kit<sup>+</sup> progenitor cells. As shown in Figure 1, both of these populations were sensitive to growth inhibition by imatinib *in vitro*. Analysis of CFU-growth 9 days after incubation using an inverted microscope showed that already at the concentration of 0.5  $\mu$ M a reduction in colony formation became evident. Exposure of cells to increasing doses of imatinib (2.0 and 5.0  $\mu$ M) revealed effective inhibition of CFU growth. Overall, proliferation of the more committed Sca1<sup>-</sup>/c-kit<sup>+</sup> cells was less susceptible to inhibition by imatinib as com-



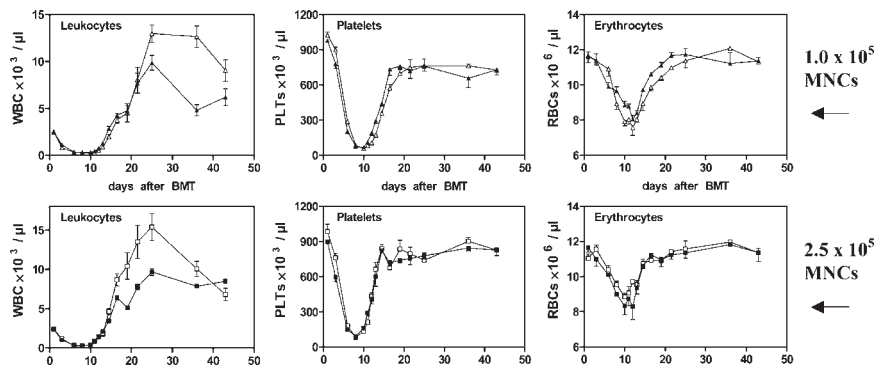
**Figure 1** Methylcellulose assay illustrating the influence of imatinib upon murine hematopoietic stem/progenitor cells *in vitro*. After flow cytometric sorting of freshly harvested murine BMCs using c-kit-PE and Sca1-FITC antibodies, 800 progenitor cells each were cultured in methylcellulose medium (Methocult GF M3434). Different concentrations of imatinib (0.0  $\mu$ M, 0.5  $\mu$ M, 2.0  $\mu$ M or 5.0  $\mu$ M) were added to evaluate imatinib dependent CFU-growth. Total number of CFUs was determined 9 days after incubation.

pared to Sca1<sup>+</sup>/c-kit<sup>+</sup> cells (28.1% Sca1<sup>-</sup>/c-kit<sup>+</sup> colonies and 9.5% Sca1<sup>+</sup>/c-kit<sup>+</sup> colonies at 5.0  $\mu$ M imatinib compared to no treatment). The reduction in size (data not shown) as well as colony number underscores the antiproliferative effect of imatinib on hematopoietic progenitor cells *in vitro* in accordance with earlier studies.

### Imatinib does not delay engraftment after syngeneic BMT in mice

Next we sought to determine whether the inhibitory effects of imatinib on hematopoietic cells *in vitro* were also relevant in an *in vivo* setting. Sixteen lethally irradiated (800 rad total dose) recipient mice of identical age were transplanted with either  $1 \times 10^5$  or  $2.5 \times 10^5$  mononuclear bone marrow cells (BMCs) from 5-FU-treated male donor mice. Administration of imatinib or placebo was begun 7 days prior to transplantation in order to ensure adequate *in vivo* blood levels of imatinib in the recipient mice. Our imatinib treatment regimen, in comparison to doses of 300–1000 mg/day (3–15 mg/kg/day) given to patients in clinical trials, consisted of 25 mg/kg every 12 h applied via gavage. The dose of imatinib was chosen according to previous papers and our experience in investigating molecular mechanisms of imatinib resistance using a murine retroviral CML transduction and transplantation model.<sup>17,26,27</sup> Twice daily administration of imatinib in this animal model is necessary since the half-life of imatinib in mice is shorter than in humans.<sup>26</sup> CML mice with elevated blood counts responded to this dose of imatinib after 3–5 days and rapidly normalized white blood cell counts (data not shown).

Interestingly, within both groups no significantly delayed hematopoietic short-term reconstitution was observed when comparing eight mice receiving 1.0 mg imatinib (50 mg/kg/day) with eight control mice (Figure 2). All animals regained normal peripheral blood cell counts after 14 to 16 days (platelets, erythrocytes) and 16 to 18 days (leukocytes).



**Figure 2** Influence of imatinib treatment on hematopoietic recovery in 16 Balb/C-mice syngeneically transplanted with two different doses of native mononuclear BMCs (MNCs). Donor mice were given 3 mg 5-FU 5 days before bone marrow harvest and transplantation. After lethal irradiation (800 rad) recipient mice were injected with either 100 000 cells and treated with imatinib ( $\blacktriangle$ ) or given H<sub>2</sub>O ( $\triangle$ ) (upper panel) or were injected with 250 000 cells and treated with imatinib ( $\blacksquare$ ) or given H<sub>2</sub>O ( $\square$ ) (lower panel). Administration of imatinib (H<sub>2</sub>O respectively) was started 7 days prior to transplantation.

Recovery kinetics showed that the time of reconstitution depended mainly on the number of transplanted cells, which is in line with previous studies from other groups<sup>28</sup> (Figure 2, compare upper and lower graphs). On average, transplantation of  $1 \times 10^5$  compared to  $2.5 \times 10^5$  mononuclear BMCs led to a 2 day delayed reconstitution.

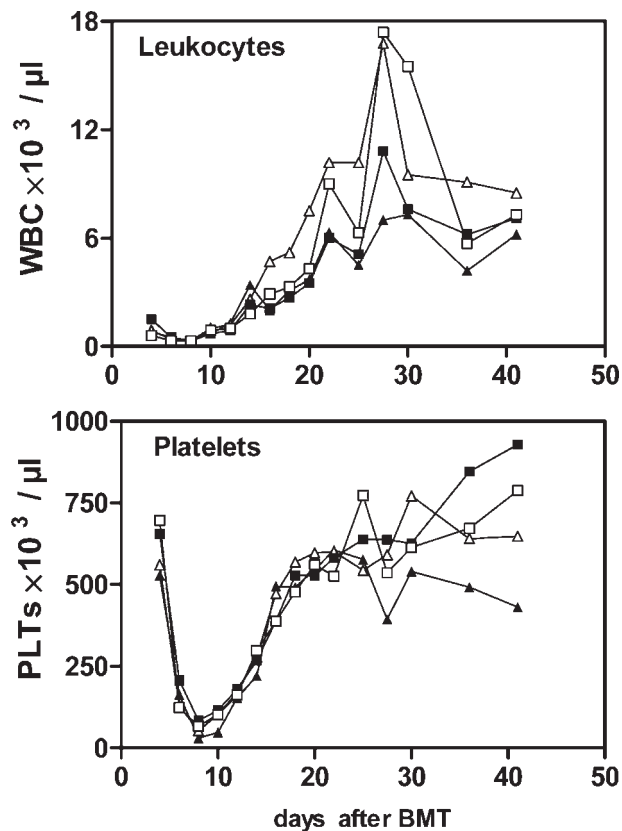
#### *Long-term engraftment is not affected by administration of imatinib*

Analysis of blood counts up to 43 days post transplant showed normal cell numbers in all transplanted mice, indicating that sufficient long-term engraftment had been achieved. We next performed secondary transplantation of mononuclear BMCs from primary imatinib treated or control mice to confirm successful long-term engraftment and to investigate potential cumulative effects of imatinib treatment on stem cell survival (Figure 3). For the reconstitution of four female lethally irradiated syngeneic Balb/C-mice approximately  $1 \times 10^6$  mononuclear BMCs from non-5-FU-treated primary animals were used. Again, the secondary transplanted Balb/C-mice showed nearly identical hematopoietic recovery independent of prior imatinib administration. In addition, we did not observe any difference in the proportion of HSC populations of four primary recipients as assessed by flow cytometry analyzing the Sca1 (Ly-6A/E), c-kit (CD117) and Thy1.2 (CD90) surface marker expression of freshly harvested BMCs (data not shown). Thus, neither short-term nor long-term hematopoietic reconstitution after syngeneic BMT was significantly influenced by the concomitant administration of imatinib.

#### **Discussion**

Although allogeneic SCT is considered the only curative approach for Ph<sup>+</sup> leukemias, a significant fraction of these patients encounters relapse. In this situation treatment with imatinib has been shown to reinduce hematologic remissions in few published cases until now.<sup>13–15</sup> New treatment approaches using imatinib concomitant with autologous or allogeneic transplantation may lead to improved outcome.

We conducted this study to determine whether hemato-



**Figure 3** Hematopoietic recovery of secondary transplanted Balb/C-mice. After lethal irradiation mice were transplanted with  $1 \times 10^6$  mononuclear BMCs from either one primary imatinib treated mouse (squares) or one H<sub>2</sub>O-control mouse (triangles). Four recipient mice were again treated with imatinib (solid symbols) or given H<sub>2</sub>O (open symbols) starting 7 days prior to transplantation. Illustrated blood curves demonstrate successful long-term engraftment independent from imatinib or H<sub>2</sub>O administration.

ietic engraftment is impaired by continued administration of imatinib during BMT. First, we chose a methylcellulose-based clonal colony assay to investigate possible inhibitory effects of imatinib on hematopoietic stem/progenitor cells as described earlier.<sup>17</sup> Our results clearly showed that sorted c-kit<sup>+</sup> hematopoietic progenitor cells are growth inhibited by imatinib *in*

*vitro*. The rate of colony formation compared to control incubated cells dramatically decreased to 9.5% and 28.1%, respectively, at 5.0  $\mu$ M imatinib. One explanation is that inhibition of the c-kit and platelet-derived growth factor receptor, two molecules also known to be effectively targeted by imatinib, may play an important role in the reduction of proliferation and differentiation in this assay. This assumption is supported by the fact that growth inhibition by imatinib in c-kit sorted cells as used in this paper was more prominent by far than in the report by Druker and colleagues<sup>17</sup> in which unsorted BMCs were used. Although this *in vitro* assay does not adequately reproduce conditions *in vivo*, these observations seemed to argue for a possible imatinib-dependent delay of hematopoietic reconstitution. Thus, we examined the influence of imatinib on bone marrow engraftment in a murine transplantation model. Interestingly, no significant difference in complete hematopoietic reconstitution and engraftment was detectable when comparing eight control mice with eight mice treated with imatinib starting at day -7. In line with previously published data, regeneration of normal blood counts mainly depended on the number of transplanted BMCs,<sup>28</sup> and the administration of 25 mg/kg imatinib every 12 h had no significant effect.

Both short- and long-term engraftment were demonstrated by repeated blood counts at least every other day over a period of at least 42 days and by flow cytometric analysis of the hematopoietic cell population after successful engraftment. In addition, secondary transplantations were performed from four primary transplanted Balb/C-mice pretreated with imatinib. Again, blood cell counts and flow cytometric analysis indicated normal hematopoietic reconstitution and successful engraftment. The serially transplanted mice regained normal blood cell counts after the same period of time. Together these data indicate that in transplanted mice engraftment and proliferation of hematopoietic stem cells was not compromised by imatinib.

Several mechanisms may be responsible for the divergent effects induced by imatinib *in vitro* and *in vivo*. The colony assays were performed using a methylcellulose culture medium containing a limited number of growth factors and cytokines and do not adequately mimic conditions *in vivo*. Furthermore, colony assays rather assess the proliferative potential of more committed progenitor cells and do not reflect stem cell function. It has recently been described that imatinib seems to inhibit committed Ph<sup>+</sup> progenitors from CML patients, but also from normal BM, more strongly than primitive progenitor cells.<sup>29</sup> Thus, the synergistic effects of hematopoietic growth factors, cytokines and the bone marrow microenvironment as well as a reduced susceptibility of early hematopoietic progenitor cells may explain the differences between the *in vitro* and *in vivo* data.

In summary, although conditions in murine BMT differ from human transplantation, our results may indicate that imatinib also does not have a negative effect on stem cell engraftment in humans. Thus, the application of imatinib in the course of allogeneic BMT or SCT could be a therapeutic regimen without suppressive influence on engraftment and hematopoietic regeneration. Further studies will be necessary to establish whether these results can be confirmed in patients.

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