

Stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-expressing leukemia cells

Ellen Weisberg,¹ Renee D. Wright,²
Douglas W. McMillin,¹ Constantine Mitsiades,¹
Arghya Ray,¹ Rosemary Barrett,¹ Sophia Adamia,¹
Richard Stone,^{1,3} Ilene Galinsky,¹
Andrew L. Kung,² and James D. Griffin¹

¹Department of Medical Oncology/Hematologic Neoplasia, Dana-Farber Cancer Institute; ²Department of Pediatric Oncology, Dana-Farber Cancer Institute and Children's Hospital;

³Department of Medicine, Harvard Medical School, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts

Abstract

Clinical studies of patients with chronic myeloid leukemia revealed that a common pattern of response is a dramatic fall in the circulating population of blast cells, with a minimal or delayed decrease in marrow blasts, suggesting a protective environment. These observations suggest that a greater understanding of the interaction of stromal cells with leukemic cells is essential. Here, we present an *in vivo* system for monitoring relative tumor accumulation in leukemic mice and residual disease in leukemic mice treated with a tyrosine kinase inhibitor and an *in vitro* system for identifying integral factors involved in stromal-mediated cytoprotection. Using the *in vivo* model, we observed high tumor burden/residual disease in tissues characterized as significant sources of hematopoiesis-promoting stroma, with bone marrow stroma most frequently showing the highest accumulation of leukemia in untreated and nilotinib-treated mice as well as partial protection of leukemic cells from the inhibitory effects of nilotinib. These studies, which showed a pattern of leukemia distribution consistent with what is observed in imatinib- and nilotinib-treated chronic myeloid leukemia patients, were followed by a more in-depth analysis of stroma-leukemia cell interactions that lead to protection of

leukemia cells from nilotinib-induced cytotoxicity. For the latter, we used the human BCR-ABL-positive cell line, KU812F, and the human bone marrow stroma cell line, HS-5, to more closely approximate the bone marrow-associated cytoprotection observed in drug-treated leukemia patients. This *in vitro* system helped to elucidate stromal-secreted viability factors that may play a role in stromal-mediated cytoprotection of tyrosine kinase inhibitor-treated leukemia cells. [Mol Cancer Ther 2008;7(5):1121–9]

Introduction

There is a growing level of interest in determining the role of the microenvironment, such as stromal cells in the marrow, in regulating growth, self-renewal, and drug resistance of leukemic stem cells. This is likely to be of more than passing interest because it appears possible that small numbers of leukemic CD34⁺ cells can persist in the marrow microenvironment of patients with chronic myeloid leukemia (CML) following years of therapy with imatinib mesylate (Gleevec, STI571; Novartis Pharma AG). Indeed, the number of leukemic stem cells that exist and that are dependent on stroma to survive is predictive of disease outcome (1).

Hematopoietic and stromal cells, along with other factors including extracellular matrix and vessels, comprise bone marrow; cell-cell interactions and growth factors influence the rate of hematopoiesis (2). The stroma of hematopoietic organs contributes to the development not only of normal hematopoietic cells but also to that of leukemia cells (3, 4). Specifically, bone marrow stroma is the source of signals, such as stem cell factor, granulocyte colony-stimulating factor, and granulocyte macrophage colony-stimulating factor, which either mediate the expansion of or prevention of the terminal differentiation of hematopoietic stem cells or support leukemia cell growth (5–11). Bone marrow stroma and stromal cell-derived soluble factors have been implicated in the long-term survival and growth of various hematologic malignancies, including precursor B-lineage acute lymphoblastic leukemia (12, 13). Bone marrow stroma has also been shown to prevent apoptosis of acute myeloid leukemia and chronic lymphocytic leukemia cells (14–16). In addition, leukemic lymphoblasts that are coupled to bone marrow stroma via gap junction communication are proposed to be held in a quiescent, nondividing state, which is believed to contribute to resistance to antimetabolic agents (17). Splenic stroma and splenocytes also play an important role in the support of the viability and proliferation of both normal and malignant hematopoiesis (18, 19).

Here, we track the progression of leukemia growth using an *in vivo* bioluminescence model of leukemia, which includes monitoring the relative degree and localization of

Received 11/26/07; revised 1/7/08; accepted 1/23/08.

Grant support: NIH grants CA66996, CA36167, and DK50654 and Leukemia and Lymphoma Society Specialized Center of Research Award. C. Mitsiades, R. Stone, A.L. Kung, and J.D. Griffin have a financial interest with Novartis Pharma AG.

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

Requests for reprints: Ellen Weisberg, Department of Medical Oncology/Hematologic Neoplasia, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: 617-632-3575; Fax: 617-632-4388. E-mail: Ellen_Weisberg@dfci.harvard.edu

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-07-2331

tumor burden in different tissue sources in untreated mice and the relative degree and localization of residual disease in mice treated with a range of doses of the novel, selective ABL tyrosine kinase inhibitor, nilotinib (AMN107; Tasisga) for varying lengths of time. Using this *in vivo* model, we show how leukemia appears to migrate to stroma-associated tissues, with the highest tumor burden accumulating in these regions. This finding mimics the trend that is observed in tyrosine kinase inhibitor-treated CML and acute myeloid leukemia patients (residual disease observed in the bone marrow stroma). For the latter half of our studies, we seek to identify the factor(s) mediating the effects of stromal-enhanced viability of leukemia growth.

Materials and Methods

Cell Lines

The human CML cell line KU812 and the HS-5 bone marrow stromal cell line were purchased from American Type Culture Collection. Murine hematopoietic 32D cells were transduced with retrovirus to express p210 BCR-ABL (32D.p210 cells; ref. 20); this cell line is rapidly lethal in

syngeneic, nonimmunosuppressed C3H mice. KU812F and 32D.p210 cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc). Luciferase-expressing cells were selected with G418 at a concentration of 1 mg/mL to produce the KU812F-luc+ and 32D.p210-luc+ cell lines, respectively.

Cell lines were cultured with 5% CO₂ at 37°C at a concentration of 2×10^5 to 5×10^5 in RPMI (Mediatech) with 10% FCS and supplemented with 1% glutamine. Transfected cell lines were cultured in medium supplemented with 1 mg/mL G418.

Chemical Compounds and Biological Reagents

Imatinib and nilotinib were synthesized by Novartis Pharma AG and, when used for *in vitro* experiments, were dissolved in DMSO to make 10 mmol/L stock solutions. Serial dilutions were then made, also in DMSO, to obtain final dilutions for cellular assays.

In vitro Proliferation Studies

For stromal rescue studies involving the KU812F cell line, we introduced luciferase into the leukemic cells so that we could specifically quantify the viable cell number using

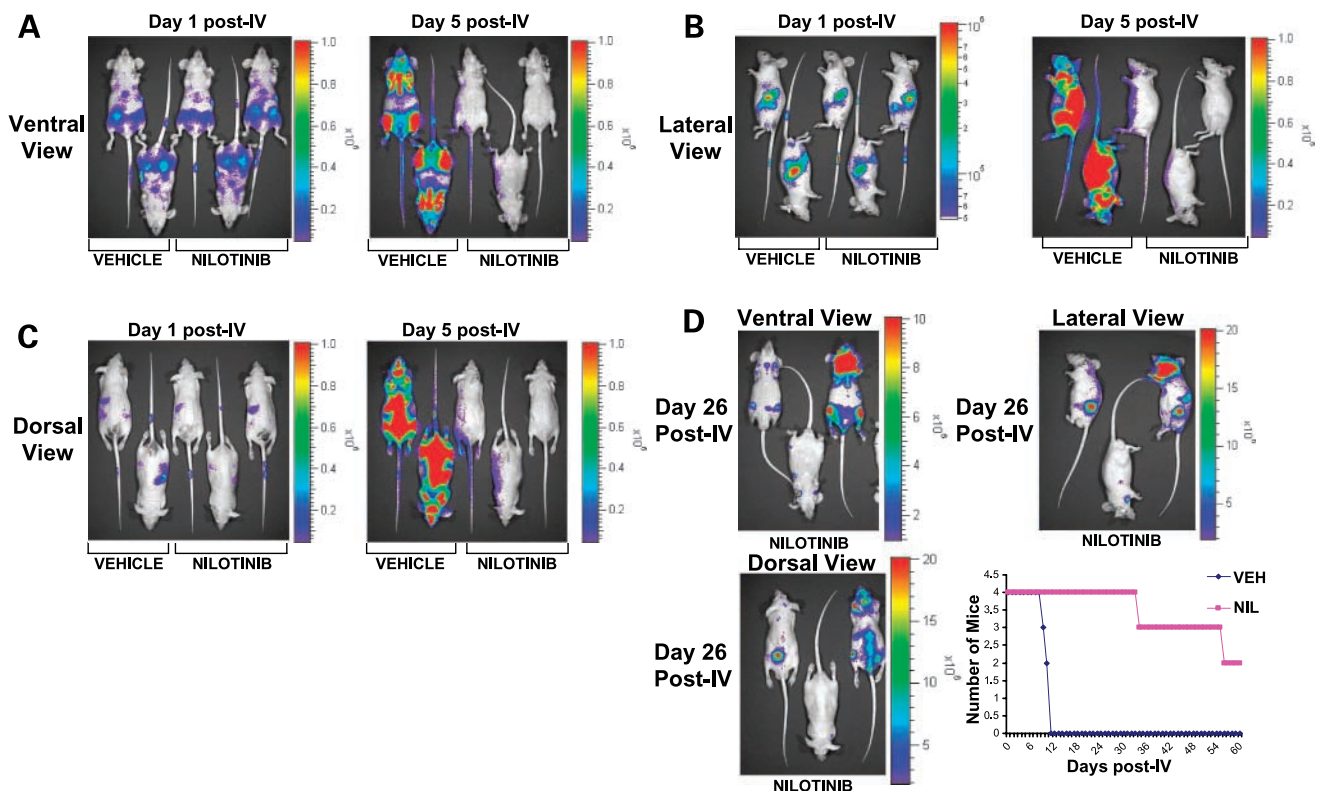
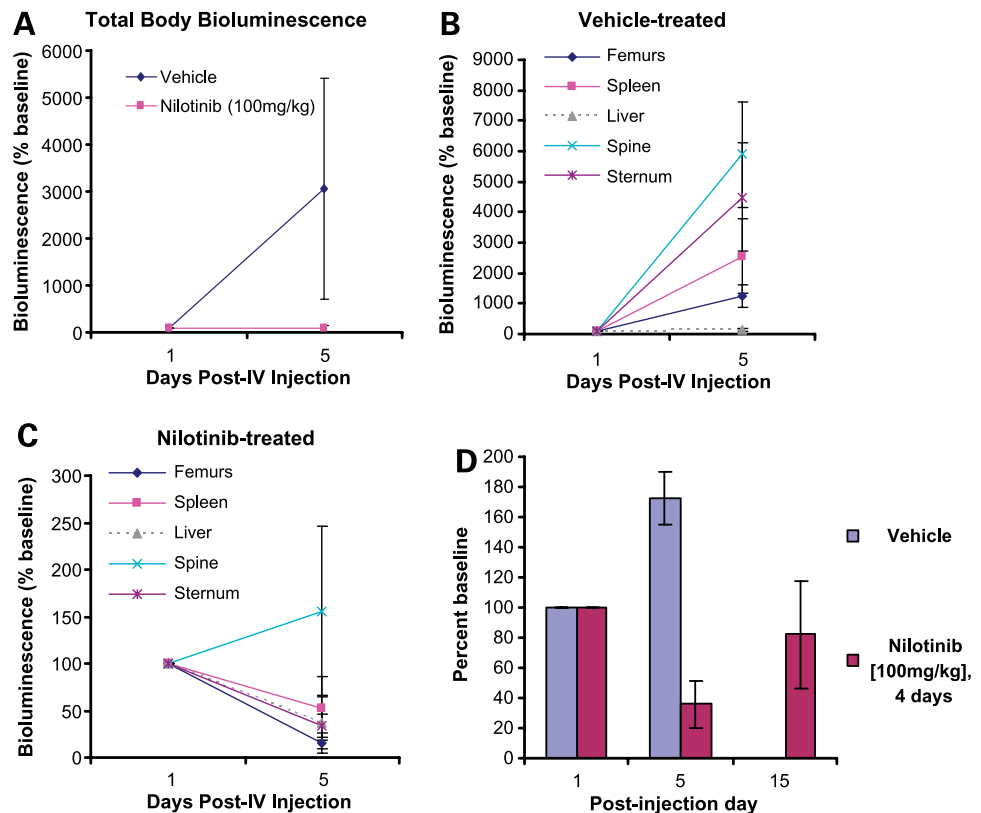


Figure 1. Leukemia growth in NCR-nude mice treated with vehicle or high-dose nilotinib. Day 1 after i.v. injection of 32D.p210-luc+ cells via tail vein injection are baseline images. NCR-nude mice were treated, starting on this first (baseline) imaging day, for 4 d with either vehicle or nilotinib (100 mg/kg). **A**, ventral view images, days 1 and 5 after i.v. injection of 32D.p210-luc+ cells. **B**, lateral view images, days 1 and 5 after i.v. injection of 32D.p210-luc+ cells. **C**, dorsal view images, days 1 and 5 after i.v. injection of 32D.p210-luc+ cells. Representative mice shown. Order of mice in **A** to **C** from left to right: VEH926, VEH929, NIL927, NIL930, NIL928. **D**, day 26 after i.v. images of nilotinib (100 mg/kg, 4 d) – treated NCR-nude mice following drug withdrawal on day 5 after i.v. injection of 32D.p210-luc+ cells. **Top left**, ventral view; **top right**, lateral view; **bottom left**, dorsal view. Order of mice in **D** from left to right: NIL927, NIL930, NIL928. **Bottom right**, survival curve for vehicle-treated and nilotinib (100 mg/kg, 4 d) – treated NCR-nude mice. Two surviving nilotinib-treated mice were sacrificed on day 61 after i.v. injection of 32D.p210-luc+ cells and were imaged 1 d later to establish baseline values. Mice were i.v. injected with 800,000 32D.p210-luc+ cells and were imaged 1 d later to establish baseline values.

Figure 2. Antileukemia drug effects and relative tumor burden in NCR-nude mice treated with high-dose nilotinib. **A**, total body bioluminescence (mean \pm SE of ventral, dorsal, and lateral views) in vehicle- and nilotinib-treated mice. **B**, relative tumor burden in vehicle-treated NCR-nude mice. **C**, relative tumor burden in nilotinib (100 mg/kg, 4 d)-treated NCR-nude mice. Data for **B** and **C** are presented as percent of each organ/area's baseline value. **D**, WBC counts (counted by hemacytometer and trypan blue exclusion following RBC lysis) in NCR-nude mice treated with vehicle or 100 mg/kg/d nilotinib for 4 d. Drug was withdrawn on day 5 after i.v., and WBC counts were taken again on day 15 after i.v. WBC counts are shown as percent of control (baseline WBC counts on day 1 after i.v. injection). $P = 0.003$ after i.v. injection day 5.



light emission. Thus, luciferase expression, which positively correlates with cell viability, was used for proliferation studies involving protection of imatinib- or nilotinib-treated KU812F-luc by stromal conditioned medium (SCM) or cytokines. Luciferase expression in these cell lines was measured by a luminometer. Approximately 10,000 HS-5 stromal cells were plated and then cultured in the absence of leukemia cells for the reported lengths of time before medium was pooled and collected and used in proliferation studies measuring protection of drug (tyrosine kinase inhibitor)-treated cells by SCM.

CML Patient Cell Studies

Frozen vials of bone marrow samples from CML (at least 50% blasts) were thawed and treated with Ficoll-Plaque (Pharmacia). The trypan blue exclusion assay has been described previously (21) and was used for proliferation studies involving CML patient samples.

Mouse Studies and *In vivo* Imaging

32D.p210 cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc) and selected with G418 at a concentration of 1 mg/mL to produce the 32D.p210-luc+ cell line. 32D.p210-luc+ cells free of *Mycoplasma* and viral contamination were washed once with HBSS (Mediatech) and resuspended in HBSS before administration to mice. Nilotinib was formulated by first dissolving powder stock in NMP to give a clear solution (stored for several days at 4°C) and diluting 10-fold in PEG300 just before administration to mice. Gavage volumes were fixed

according to the individual weights of the mice to achieve 20 or 100 mg/kg nilotinib depending on the study.

A total of 800,000 32D.p210-luc+ cells were administered via tail vein injection to male NCR-nude mice (5-6 weeks old; Taconic). Anesthetized mice were imaged 1 day after i.v. injection to generate a baseline that was used to establish treatment cohorts with matched tumor burden, and total body luminescence was measured as described previously (22). Cohorts of mice were treated with either oral administration of vehicle (10% NMP-90% PEG300) or oral administration of nilotinib (formulated as above). Mice were monitored for a period following the last imaging day and before sacrifice. Tissues were preserved in 10% formalin for histopathologic analysis and confirmation of tumor burden in vital organ tissues; tissues were also harvested to obtain cells for *ex vivo* analysis.

The low dose of 20 mg/kg nilotinib was predetermined to be subcurative *in vivo* (23) and the relatively high dose of 100 mg/kg nilotinib was chosen based on earlier findings that 75 mg/kg/d nilotinib administered over a 16-day period was curative in the majority of treated mice over an observation period of ~100 days (24).

Results

Leukemia Growth in NCR-Nude Mice Treated with Vehicle or High-Dose Nilotinib

Using a bioluminescent *in vivo* model of BCR-ABL leukemia, we investigated the growth of BCR-ABL-positive

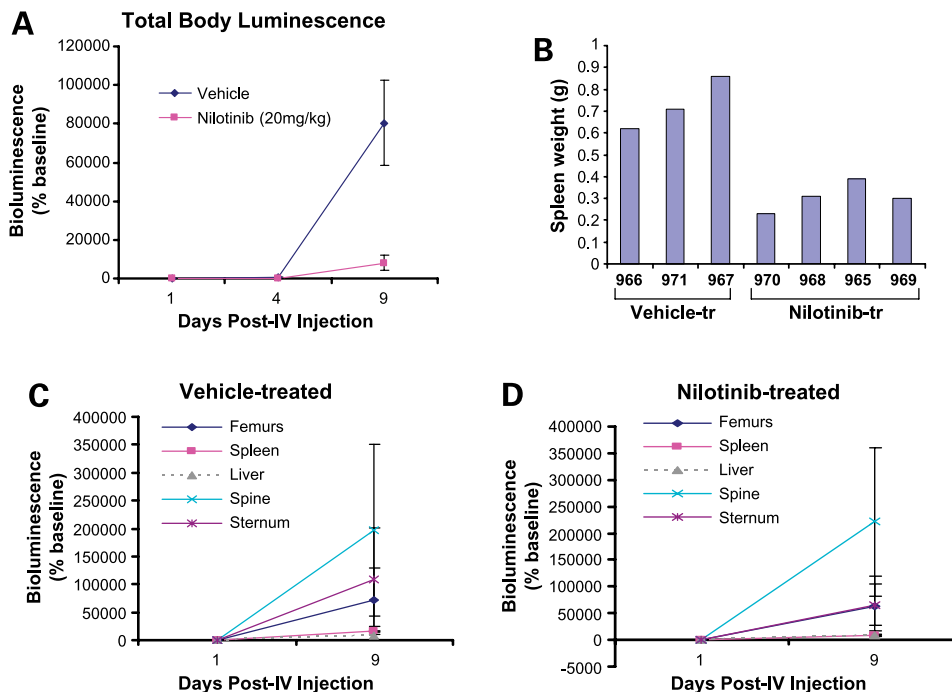


Figure 3. Antileukemia drug effects, relative tumor burden, and stromal-mediated cytoprotection in NCR-nude mice treated with low-dose nilotinib. **A**, total body luminescence: Mean \pm SE of ventral, dorsal, and lateral views of vehicle-treated and nilotinib (20 mg/kg, 8 d)-treated NCR-nude mice. **B**, spleen weights (in grams) for vehicle-treated and nilotinib-treated NCR-nude mice measured on day 16 after i.v. injection of 32D.p210-luc+ cells. **C**, relative tumor burden in vehicle-treated NCR-nude mice days 1 and 9 after i.v. injection of 32D.p210-luc+ cells. **D**, relative tumor burden in nilotinib (100 mg/kg, 4 d)-treated NCR-nude mice days 1 and 9 after i.v. injection of 32D.p210-luc+ cells. Data for **C** and **D** presented as percent of each organ/area's baseline value.

leukemia in vehicle-treated mice and the pattern of residual disease in nilotinib-treated mice. We observed highest tumor burden in areas of vehicle-treated mice that included the femurs and spleen at baseline and the femurs, sternum, spine, and spleen of vehicle-treated mice on day 4 after i.v. injection of 32D.p210-luc+ cells (Fig. 1A-C). A relatively high dose (100 mg/kg) of nilotinib was administered to mice in the drug treatment group for 4 days before drug withdrawal. Treatment with this dose caused a dramatic reduction in leukemia burden in mice, although bioluminescence values, while low, were still measurable (Figs. 1A-C and 2).

Antileukemia Drug Effects and Relative Tumor Burden in NCR-Nude Mice Treated with High-Dose Nilotinib

In addition to high-dose nilotinib treatment lowering total body luminescence in mice, it also significantly lowered WBC counts in mice by day 5 after i.v. injection (Fig. 2A and D) and led to a significant prolongation of survival compared with vehicle-treated mice (Fig. 1E). However, we observed a visually and measurably detectable recurrence of leukemia burden in the high-dose (4-day) nilotinib-treated mice on day 19 after i.v. injection of cells (Supplementary Data)⁴ and day 26 after i.v. (Fig. 1D). High tumor burden areas included the femurs, sternum, spleen, and parts of the skull (Fig. 1D). Bioluminescence was also observed in the shape, location, and orientation approximately corresponding to the shape, orientation, and anatomic location where superficial/deep

cervical lymph nodes have been identified in the mouse (Fig. 1D). WBC counts, while still lower in drug-treated mice than vehicle-treated mice on day 15 after i.v. injection of leukemia cells, were observed to rise in number in the 4-day nilotinib-treated mice with drug having been withdrawn for 10 days (Fig. 2D).

We investigated the relative tumor burden of those tissues in vehicle- and nilotinib-treated mice that either showed highest leukemia burden and are characterized by significant amounts of stroma (spine, femurs, sternum, and spleen) or were known organ targets often used as a marker of leukemia progression (spleen and liver). Interestingly, in both vehicle-treated and high-dose nilotinib-treated mice, the spine was observed to display the highest relative tumor burden (Fig. 2B and C).

Antileukemia Drug Effects and Relative Tumor Burden in NCR-Nude Mice Treated with Low-Dose Nilotinib

A relatively low dose (20 mg/kg) of nilotinib, administered for a total of 8 days, similarly lowered total body luminescence in mice and prevented the increased spleen weights observed in vehicle-treated mice (Fig. 3A and B; Supplementary Data).⁴ As with the high-dose nilotinib study, the highest tumor burdens as measured by bioluminescence were observed in the femurs, sternum, spine, and spleens of vehicle-treated and low-dose nilotinib-treated mice (Supplementary Data).⁴ The relative tumor burden in vehicle-treated and low-dose nilotinib-treated mice was again observed to be highest in the spine on days 4 and 9 after i.v. injection of 32D.p210-luc+ cells followed by femurs (Fig. 3C and D; Supplementary Data).⁴

In another study, we administered 20 mg/kg nilotinib to mice for a total of 3 days. As was observed in the other

⁴Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

in vivo imaging studies, the highest tumor burdens as measured by bioluminescence were again observed in the femurs, sternum, spine, and spleens of vehicle-treated and low-dose nilotinib-treated mice (Supplementary Data).⁴ The relative tumor burden in low-dose nilotinib-treated mice was again observed to be highest in the spine on day 4 after *i.v.* injection of 32D.p210-luc+ cells (Supplementary Data).⁴ However, the spleen showed the highest relative tumor burden in vehicle-treated mice compared with spine, liver, and femurs (Supplementary Data).⁴ The overall similarity in the results obtained with high- and low-dose nilotinib suggests that residual disease in drug-treated mice is associated with tissues bearing high stroma content regardless of the efficacy of the drug treatment.

Effects of SCM on the Proliferation of BCR-ABL-Expressing Cells

We investigated whether stromal-derived, secreted factor(s) could increase BCR-ABL-positive cell proliferation and/or protect cells against the inhibitory effects of selective tyrosine kinase inhibitors. To achieve this, culture medium pooled and collected from HS-5 stromal cells cultured for 1 week was tested with KU812F-luc+ cells incubated in the absence and presence of imatinib and nilotinib, respectively. We observed that SCM, diluted with FCS-containing RPMI across a range of concentrations (10-75%), was able to partially protect KU812F-luc+ cells from the inhibitory effects of both imatinib and nilotinib, respectively (Fig. 4A and B). In addition, a bone marrow sample derived from a CML-MBC (myeloid blast crisis) patient (not imatinib-naïve) was protected by the inhibitory effects of imatinib when cultured in the presence of SCM (Fig. 4C). These results suggest that stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-positive cells appears to involve viability signals in the form of one or more secreted growth factors.

Effects of Human Stroma-Derived Cytokines on the Proliferation of BCR-ABL-Expressing Cells

To explore this possibility further, we randomly screened the effects of stromal-derived factor-1 (10 ng/mL), which is constitutively produced by bone marrow stromal cells and behaves as a chemoattractant supporting the homing of stem cells, as well as a mixture of cytokines shown previously to be secreted at high concentrations in SCM derived from HS-5 stromal cells [including stem cell factor, interleukin (IL)-6, IL-8, IL-11, macrophage colony-stimulating factor, and granulocyte macrophage colony-stimulating factor; each at a concentration of 10 ng/mL; ref. 25], to determine if a cocktail of these cytokines would be able to protect imatinib-treated, BCR-ABL-positive cells from the inhibitory effects of the drug. We found the cytokine cocktail was able to potentiate the growth of untreated KU812F-luc+ cells and to partially protect imatinib-treated KU812F-luc+ cells to a similar extent as SCM across a range of concentrations (10-50%), which was collected and pooled from stromal cells cultured for 22 days (Fig. 5A and C; Supplementary Data).⁴ When each cytokine (at 10 ng/mL) was compared with the mixture of cytokines, we observed varying potentiation of KU812F-luc+ cell growth and

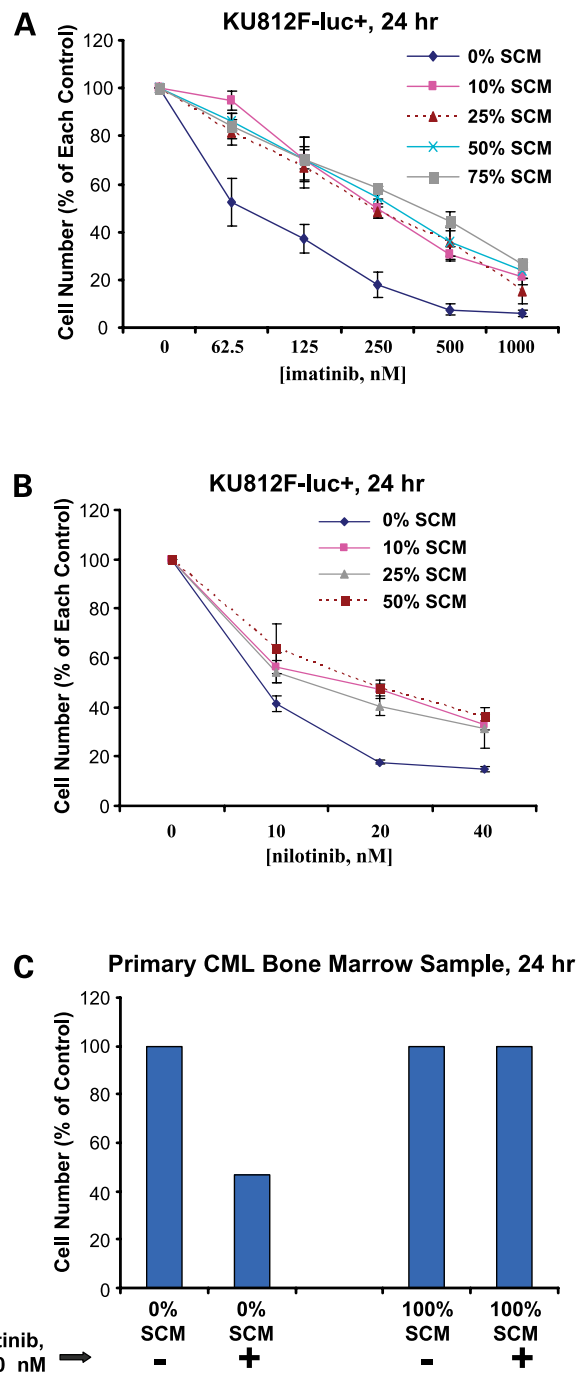


Figure 4. Effects of SCM on the proliferation of BCR-ABL-expressing cells. Treatment of KU812F-luc+ cells for 24 h with imatinib (**A**) or nilotinib (**B**) in the absence and presence of SCM at varying concentrations (collected and pooled from HS-5 stroma cultured for a total of 7 d). Cell number for drug-treated KU812F-luc+ cells cultured in the absence of SCM is shown as the percent of the no SCM control. Cell number for drug-treated cells cultured in the presence of each respective percentage of SCM is shown as the percent of each respective percentage of SCM control. Samples for this experiment were set up in triplicate. Bars, SE. **C**, treatment of a primary bone marrow CML-MBC patient sample (progressed *s/p* Gleevec, HHT/ara-C) for 24 h with imatinib in the absence and presence of SCM (medium conditioned for 7 d in the presence of stromal cells).

protective capacities of the individual cytokines, all of which were generally less than that of the cytokine cocktail (Fig. 5B and D). These results suggest that two or more of the tested cytokines are likely required in combination to achieve the maximum protection observed when drug-treated KU812F-luc+ cells are cocultured with SCM or cytokine cocktail.

To further investigate this, we tested the combination of paired cytokines to assess the extent of the protective effects of each pair against imatinib-treated KU812F-luc+ cells compared with the cytokine cocktail. We did observe varying degrees of protection with the different cytokine pairings; however, as was observed with individual cytokines, none of the paired cytokines were able to protect drug-treated cells to the full extent of the cytokine cocktail (Fig. 6).

Discussion

Investigators have been actively exploring the relationship between malignant cells and the bone marrow microenvironment to better understand the source and nature of viability-promoting factors that can aid in disease progression. A recent study used a stromal cell system, derived via bone biopsy, to investigate the interactions between chronic lymphocytic leukemia B cells, stroma cells, and stroma-secreted factors that protect the leukemic cells from naturally occurring and drug-induced apoptosis (26).

In the present study, we monitored leukemia progression *in vivo* and noted that a variety of tissues showed high tumor accumulation: those characterized by high levels of bone marrow stroma (spine, sternum, and skull) and those characterized by hematopoiesis-supporting stroma

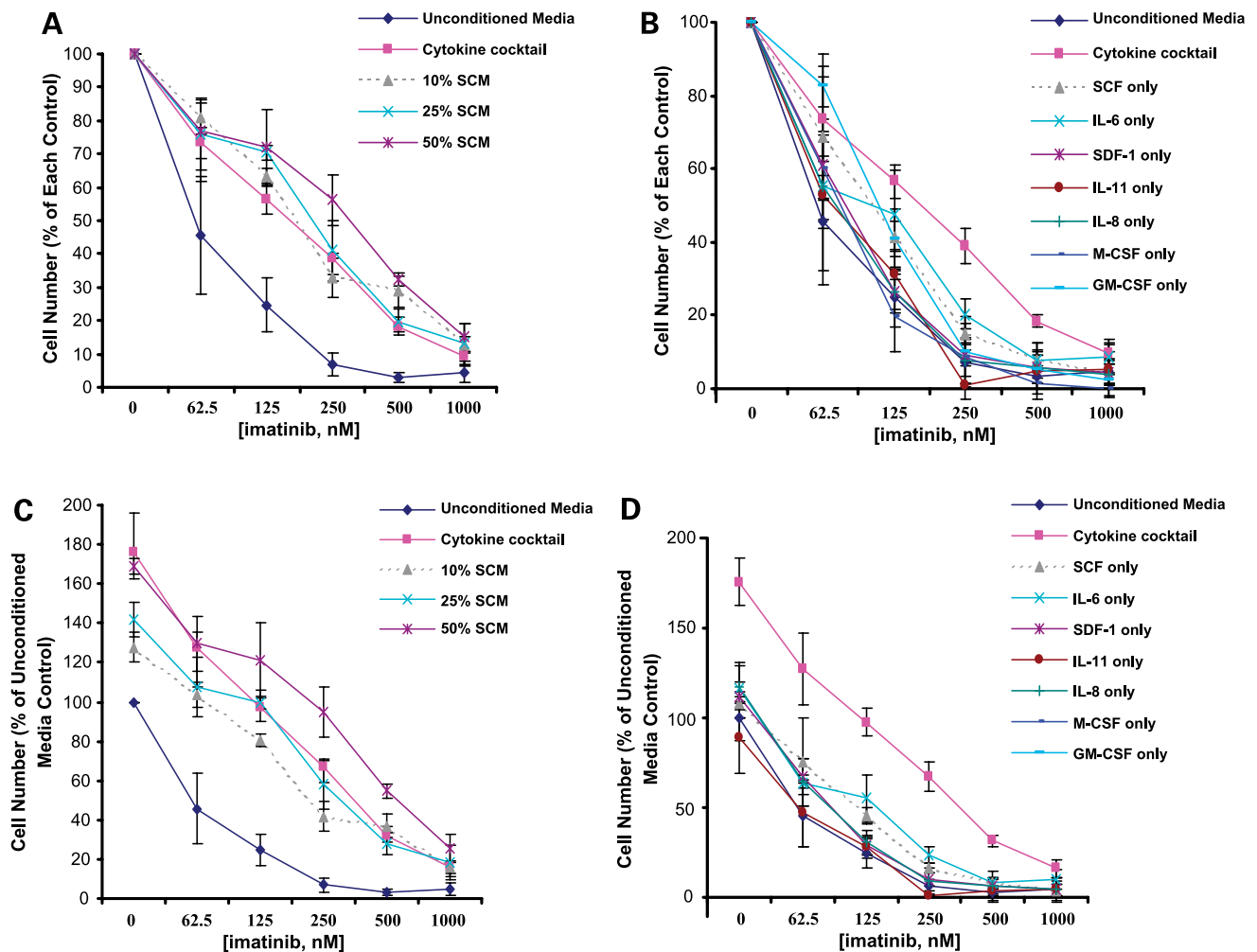


Figure 5. Effects of human stroma-derived cytokines on the proliferation of BCR-ABL-expressing cells. **A**, 24-h treatment of KU812F-luc+ cells with imatinib in the presence and absence of cytokine cocktail or SCM (conditioned for 22 d). Cell number for drug-treated KU812F-luc+ cells cultured in the absence of conditioned medium is shown as the percent of the unconditioned medium control, whereas cell number for drug-treated cells cultured in the presence of conditioned medium is shown as the percent of the conditioned medium control. Samples for this experiment were set up in triplicate. Bars, SE. **B**, 24-h treatment of KU812F-luc+ cells with imatinib in the presence and absence of cytokine cocktail (10 ng/mL of each cytokine pooled together) versus individual cytokines (10 ng/mL each). Values are presented as percent of each respective control. Samples for this experiment were set up in triplicate. Bars, SE. **C** and **D**, same as data shown in **A** and **B**, respectively, except cell number for drug-treated cells cultured in the absence and presence of conditioned medium, cytokine cocktail, or individual cytokines is shown as the percent of the unconditioned medium control.

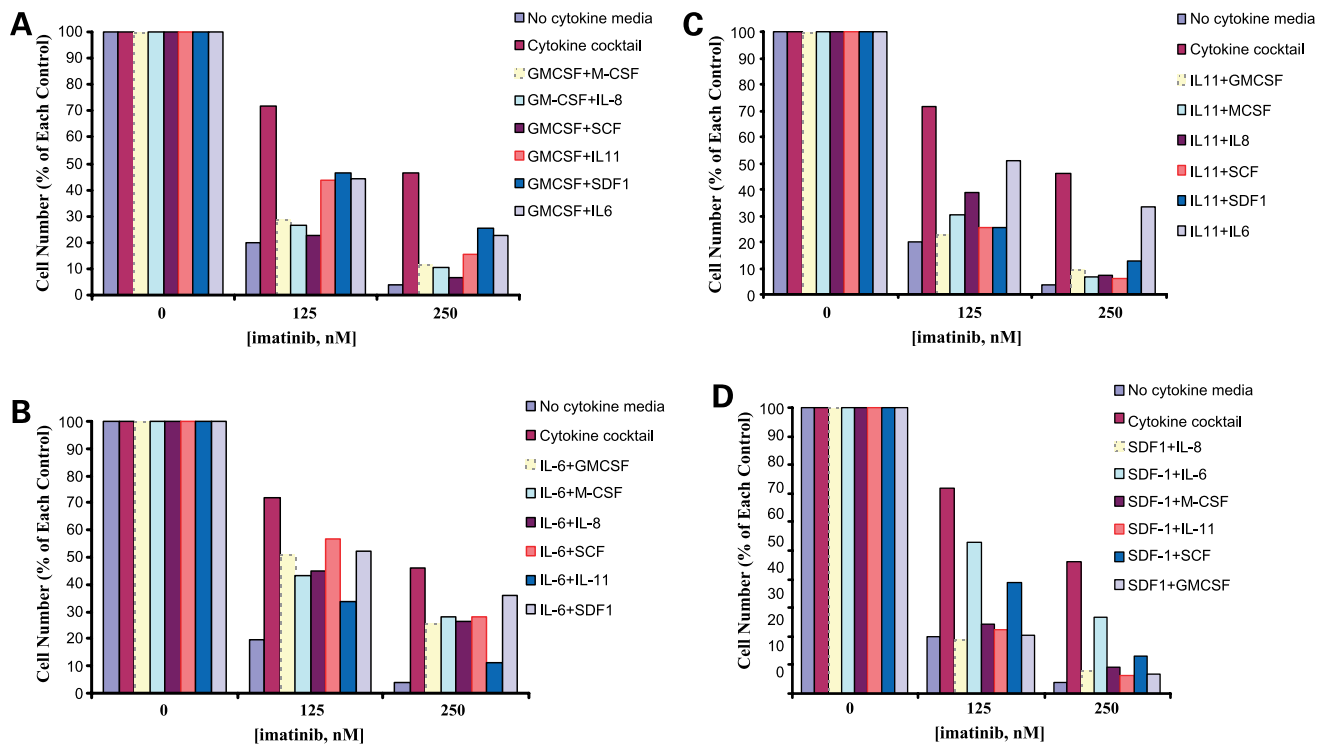


Figure 6. Effects of paired human stroma-derived cytokines on the proliferation of BCR-ABL-expressing cells. KU812F-luc⁺ cells treated for 24 h with imatinib in the absence and presence of paired cytokines. Cell number for drug-treated KU812F-luc⁺ cells cultured in the absence of cytokines is shown as the percent of the no cytokine control, whereas cell number for drug-treated cells cultured in the presence of cytokines is shown as the percent of the respective cytokine control. **A**, imatinib treatment of KU812F-luc⁺ cells in the absence and presence of individual cytokines paired with granulocyte macrophage colony-stimulating factor (10 ng/mL). **B**, imatinib treatment of KU812F-luc⁺ cells in the absence and presence of individual cytokines paired with IL-6 (10 ng/mL). **C**, imatinib treatment of KU812F-luc⁺ cells in the absence and presence of individual cytokines paired with IL-11 (10 ng/mL). **D**, imatinib treatment of KU812F-luc⁺ cells in the absence and presence of individual cytokines paired with stromal-derived factor-1 (10 ng/mL).

(spleen). These same tissues showed a high degree of residual disease in drug-treated mice, both acutely as in the case of relatively low-dose (20 mg/kg) nilotinib-treated mice observed shortly after drug withdrawal, and chronically, as in the case of relatively high-dose (100 mg/kg) nilotinib-treated mice observed several weeks following drug withdrawal. These data are consistent with the findings of others that show movement of leukemia cells, including acute lymphoblastic leukemia and chronic lymphocytic leukemia, into the stroma of bone marrow (27–29).

Although the spine of nilotinib-treated mice was found to consistently show the highest relative tumor burden when compared with other high tumor burden tissues, such as spleen, femurs, and liver, the highest relative tumor burden in vehicle-treated mice was observed to be in both the spleen and the spine. This finding may be in part explained by the fact that soluble growth factors and cytokines produced by splenic stroma have been indicated in hematopoiesis from bone marrow (19), and viability signals provided by diseased splenocytes contribute to the survival and expansion of leukemic cells (18). These reports suggest that the spleen, like bone marrow, may be a significant source of viability signals that have the ability to promote leukemic cell growth.

The stromal compartment of the spleen influences the proliferation and viability of both normal and diseased hematopoietic cells. Studies have been done that support the idea that splenic stroma supports the development of WBC, such as dendritic cells. Dendritic cell hematopoiesis is supported by long-term splenic stroma cultures (30), and dendritic cell development is supported by splenic stroma and stroma-derived growth factors (31). The differentiation of dendritic cells can be influenced by endothelial-like splenic stromal cells (32).

The splenic microenvironment has also been implicated in promotion of the viability and expansion of transformed cells. Indeed, enlargement of the spleen, or splenomegaly, characterizes many different hematologic malignancies, such as CML, especially during the later stages of the disease (33). *In vivo*, spleen removal from erythroleukemic mice was observed to prolong survival (18). Furthermore, the *in vitro* proliferation of primary erythroleukemic blast cells cocultured in the presence of either leukemic-derived spleen cells or the conditioned medium derived from such cells was enhanced compared with the proliferation of primary erythroleukemic cells cocultured with normal splenocytes, with elevated secretion by the diseased splenocytes of viability factors including IL-6, IL-12p70,

IL-2, macrophage chemoattractant protein-5, vascular endothelial growth factor-A, soluble tumor necrosis factor receptor-1, and tumor necrosis factor- α (18).

Both medium conditioned by human stromal cells and a cocktail of cytokines secreted in high concentrations by stroma are able to partially protect BCR-ABL-expressing cells from the inhibitory effects of tyrosine kinase inhibitors, such as imatinib and nilotinib, on cellular proliferation. These findings are consistent with those of others, as cytokines such as IL-6 and granulocyte macrophage colony-stimulating factor have been found to protect myeloid leukemia cells from chemotherapy-induced apoptosis (34).

Unlike the present findings with BCR-ABL-positive leukemia, not all hematologic malignancies are able to be rescued from apoptosis by secreted factors in the absence of direct contact with stromal cells. For example, a need for direct bone marrow fibroblast cell-leukemic cell interaction was observed for protection of acute myeloid leukemia cells from apoptosis (35, 36) and for protection of B-lineage acute lymphoblastic leukemia cells from chemotherapy-induced apoptosis (37). Similarly, protection of myeloma cells from drug-induced apoptosis is dependent on both adhesion between bone marrow stromal cells and myeloma cells and soluble factors induced by the cell-cell interaction (38). Finally, the adherence of chronic lymphocytic leukemia cells to bone marrow stromal cell layers was necessary for their protection from apoptosis (39).

Our results suggest that significant reservoirs for tumor expansion and accumulation appear to be tissues characterized by stroma having the ability to support normal hematopoietic and malignant stem cell development. In addition, we found that stromal cells secrete cytokines having the ability to partially rescue BCR-ABL-expressing cells from the cytotoxic effects of protein tyrosine kinase inhibitors such as imatinib and nilotinib.

In summary, we present a novel, qualitative, and quantitative *in vivo* approach to tracking tumor progression. We also identify factors that may play a role in stromal-mediated cytoprotection from antileukemia agents. The panel of cytokines that we identified as collectively mimicking the cytoprotective effects of stroma offers significant insight into putative factors that could serve as molecular targets to improve the efficacy of existing therapies and minimize residual disease in patients. It is therefore anticipated that this information may be used in the development of new strategies to override stromal-mediated chemoresistance.

Disclosure of Potential Conflicts of Interest

E. Weisberg, A. Ray, R. Barrett, R. Stone, A.L. Kung, and J.D. Griffin: Novartis grant support. R. Stone: Bristol Myers Squibb grant support. J.D. Griffin: Novartis employee. R. Stone and C. Mitsiades: Bristol Myers Squibb honoraria. C. Mitsiades: Novartis honoraria. R.D. Wright, D.W. McMillin, S. Adamia, and I. Galinsky: no potential conflicts of interest were disclosed.

References

1. Kumagai M, Manabe A, Pui CH, et al. Stroma-supported culture in childhood B-lineage acute lymphoblastic leukemia cells predicts treatment outcome. *J Clin Invest* 1996;97:755–60.
2. Charbord P, Tavian M, Humeau L, Peault B. Early ontogeny of the

human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. *Blood* 1996;87:4109–19.

3. Rafii S, Mohle R, Shapiro F, Frey BM, Moore MA. Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma* 1997;27:375–86.
4. Litwin C, Leong KG, Zapf R, Sutherland H, Naiman SC, Karsan A. Role of the microenvironment in promoting angiogenesis in acute myeloid leukemia. *Am J Hematol* 2002;70:22–30.
5. Verfaillie CM. Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. *Blood* 1993;82:2045–53.
6. Liesveld JL, Harbol AW, Abboud CN. Stem cell factor and stromal cell co-culture prevent apoptosis in a subculture of the megakaryoblastic cell line, UT-7. *Leuk Res* 1996;20:591–600.
7. Harrison PR, Nibbs RJ, Bartholomew C, et al. Molecular mechanisms involved in long-term maintenance of erythroleukaemia cells by stromal cells. *Leukemia* 1997;11:474–7.
8. Breems DA, Blokland EA, Ploemacher RE. Stroma-conditioned media improve expansion of human primitive hematopoietic stem cells and progenitor cells. *Leukemia* 1997;11:142–50.
9. O'Prey J, Leslie N, Itoh K, Ostertag W, Bartholomew C, Harrison PR. Both stroma and stem cell factor maintain long-term growth of ELM erythroleukemia cells, but only stroma prevents erythroid differentiation in response to erythropoietin and interleukin-3. *Blood* 1998;91:1548–55.
10. Leslie NR, O'Prey J, Bartholomew C, Harrison PR. An activating mutation in the kit receptor abolishes the stroma requirement for growth of ELM erythroleukemia cells, but does not prevent their differentiation in response to erythropoietin. *Blood* 1998;92:4798–807.
11. Shih CC, Hu MC, Hu J, Medeiros J, Forman SJ. Long-term *ex vivo* maintenance and expansion of transplantable human hematopoietic stem cells. *Blood* 1999;94:1623–36.
12. Bradstock K, Bianchi A, Makrynikola V, Filshie R, Gottlieb D. Long-term survival and proliferation of precursor-B acute lymphoblastic leukemia cells on human bone marrow stroma. *Leukemia* 1996;10:813–20.
13. Ashley DM, Bol SJ, Kannourakis G. Human bone marrow stromal cell contact and soluble factors have different effects on the survival and proliferation of paediatric B-lineage acute lymphoblastic leukaemic blasts. *Leuk Res* 1994;18:337–46.
14. Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. *Blood* 1998;91:2387–96.
15. Lagneaux L, Delforge A, De Bruyn C, Bernier MO, Bron D. Adhesion to bone marrow stroma inhibits apoptosis of chronic lymphocytic leukemia cells. *Leuk Lymphoma* 1999;35:445–53.
16. Konopleva M, Konoplev S, Hu W, Zaritsky AY, Afanasiev BV, Andreeff M. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* 2002;16:1713–24.
17. Paraguassú-Braga FH, Borojevic R, Bouzas LF, Barcinski MA, Bonomo A. Bone marrow stroma inhibits proliferation and apoptosis in leukemic cells through gap junction-mediated cell communication. *Cell Death Differ* 2003;10:1101–8.
18. Shaked Y, Cervi D, Neuman M, et al. The splenic microenvironment is a source of proangiogenesis/inflammatory mediators accelerating the expansion of murine erythroleukemic cells. *Blood* 2005;105:4500–7.
19. Despars G, O'Neill HC. Splenic endothelial cell lines support development of dendritic cells from bone marrow. *Stem Cells* 2006;24:1496–504.
20. Matulonis U, Salgia R, Okuda K, Druker B, Griffin JD. IL-3 and p210 BCR-ABL activate both unique and overlapping pathways of signal transduction in a factor-dependent myeloid cell line. *Exp Hematol* 1993;21:1460–6.
21. Weisberg E, Boulton C, Kelly LM, et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitors PKC412. *Cancer Cell* 2002;1:433–43.
22. Armstrong SA, Kung AL, Mabon ME, et al. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell* 2003;3:173–83.
23. Weisberg E, Catley L, Wright RD, et al. Beneficial effects of combining nilotinib and imatinib in preclinical models of BCR-ABL+ leukemias. *Blood* 2007;109:2112–20.

24. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* 2005;7:129–41.
25. Roecklein BA, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood* 1995;85:997–1005.
26. Kay NE, Shanafelt TD, Strege AK, Lee YK, Bone ND, Raza A. Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an “angiogenic switch.” *Leuk Res* 2007;31:899–906.
27. Burger JA, Kipps TJ. Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. *Leuk Lymphoma* 2002;43:461–6.
28. Miyake K, Hasunuma Y, Yagita H, Kimoto M. Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture. *J Cell Biol* 1992;119:653–62.
29. Makrynikola V, Bianchi A, Bradstock K, Gottlieb D, Hewson J. Migration of acute lymphoblastic leukemia into human bone marrow stroma. *Leukemia* 1994;8:1734–43.
30. Ni K, O’Neill HC. Long-term stromal cultures produce dendritic-like cells. *Br J Haematol* 1997;97:710–25.
31. Wilson HL, Ni K, O’Neill HC. Proliferation of dendritic cell progenitors in long term culture is not dependent on granulocyte macrophage-colony stimulating factor. *Exp Hematol* 2000;28:193–202.
32. Zhang M, Tang H, Guo Z, et al. Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. *Nat Immunol* 2004;5:1124–33.
33. Rosenthal DS. Hodgkin’s disease and non-Hodgkin’s lymphomas. In: Murphy GP, Lenard RE, editors. 2nd ed. Atlanta (GA): American Cancer Society; 1995; p. 451–85.
34. Lotem J, Sachs L. Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor β 1 and cancer chemotherapy compounds in myeloid leukemic cells. *Blood* 1992;80:1750–7.
35. Garrido SM, Appelbaum FR, Willman CL, Banker DE. Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). *Exp Hematol* 2001;29:448–57.
36. Bendall LJ, Daniel A, Kortlepel K, Gottlieb DJ. Bone marrow adherent layers inhibit apoptosis of acute myeloid leukemia cells. *Exp Hematol* 1994;22:1252–60.
37. Mudry RE, Fortney JE, York T, Hall BM, Gibston LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. *Blood* 2000;96:1926–32.
38. Nefedova Y, Landowski TH, Dalton WS. Bone marrow stromal-derived soluble factors and direct cell contact contribute to *de novo* drug resistance of myeloma cells by distinct mechanisms. *Leukemia* 2003;17:1175–82.
39. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells *in vitro*. *Br J Haematol* 1996;92:97–103.

Molecular Cancer Therapeutics

Stromal-mediated protection of tyrosine kinase inhibitor-treated BCR-ABL-expressing leukemia cells

Ellen Weisberg, Renee D. Wright, Douglas W. McMillin, et al.

Mol Cancer Ther 2008;7:1121-1129. Published OnlineFirst April 29, 2008.

Updated version Access the most recent version of this article at:
doi:[10.1158/1535-7163.MCT-07-2331](https://doi.org/10.1158/1535-7163.MCT-07-2331)

Supplementary Material Access the most recent supplemental material at:
<http://mct.aacrjournals.org/content/suppl/2008/07/18/1535-7163.MCT-07-2331.DC1>

Cited articles This article cites 38 articles, 12 of which you can access for free at:
<http://mct.aacrjournals.org/content/7/5/1121.full#ref-list-1>

Citing articles This article has been cited by 11 HighWire-hosted articles. Access the articles at:
<http://mct.aacrjournals.org/content/7/5/1121.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

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

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/7/5/1121>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.