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Effective Targeting of Quiescent Chronic Myelogenous Leukemia Stem Cells by Histone Deacetylase Inhibitors in Combination with Imatinib Mesylate

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SUMMARY

Imatinib mesylate (IM) induces remission in chronic myelogenous leukemia (CML) patients but does not eliminate leukemia stem cells (LSCs), which remain a potential source of relapse. Here we investigated the ability of HDAC inhibitors (HDACis) to target CML stem cells. Treatment with HDACis combined with IM effectively induced apoptosis in quiescent CML progenitors resistant to elimination by IM alone, and eliminated CML stem cells capable of engrafting immunodeficient mice. In vivo administration of HDACis with IM markedly diminished LSCs in a transgenic mouse model of CML. The interaction of IM and HDACis inhibited genes regulating hematopoietic stem cell maintenance and survival. HDACi treatment represents an effective strategy to target LSCs in CML patients receiving tyrosine kinase inhibitors.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a lethal hematological malignancy resulting from transformation of a primitive hematopoietic cell by the *BCR-ABL* oncogene (Sawyers, 1999). Leukemia-initiating cells or leukemia stem cells (LSCs) in CML share several properties with normal hematopoietic stem cells (HSCs), including the ability to regenerate multilineage hematopoiesis and quiescence (Holyoake et al., 1999; Wang et al., 1998). Progeny of transformed stem cells have a proliferative advantage over normal hematopoietic cells, allowing the Philadelphia (Ph)–positive clone to displace residual normal hematopoiesis. Without treatment, CML progresses from a chronic phase (CP) to an accelerated phase (AP) and terminal blast crisis (BC). Deregulated tyrosine kinase activity of the BCR-ABL protein plays an important role in CML pathogenesis. Treatment with BCR-ABL tyrosine kinase inhibitors (TKI) reverses the proliferative advantage of CML progenitors, inducing remission and allowing regrowth of normal hematopoietic cells. The BCR-ABL kinase inhibitor imatinib mesylate (IM, Gleevec) has emerged as the first-line treatment for patients with CML (Druker et al., 2001; O'Brien et al., 2003). Most patients with CP CML achieve complete cytogenetic response (CCR) with IM treatment and demonstrate major reductions in *BCR-ABL* transcript levels as assessed by real-time quantitative RT-PCR (Q-PCR) (Hughes et al., 2003). However, there is evidence that primitive leukemia stem and progenitor cells are retained in patients achieving remission with IM treatment (Bhatia et al., 2003). Disease

Significance

Chronic myelogenous leukemia (CML) results from transformation of a hematopoietic stem cell by the *BCR-ABL* gene. The BCR-ABL tyrosine kinase inhibitor imatinib mesylate (IM) is effective in inducing remissions and improving survival in patients with CML but does not eliminate leukemia stem cells (LSCs). Patients need continued treatment to prevent disease relapse and strategies to eliminate residual LSCs are required. Our studies indicate that treatment with the histone deacetylase inhibitors (HDACis) combined with IM is effective in inducing apoptosis in CML LSCs that resist elimination by IM alone. Several HDACis are in clinical development, and our studies support clinical trials of HDACis in combination with tyrosine kinase inhibitors to eliminate LSCs in patients with CML.

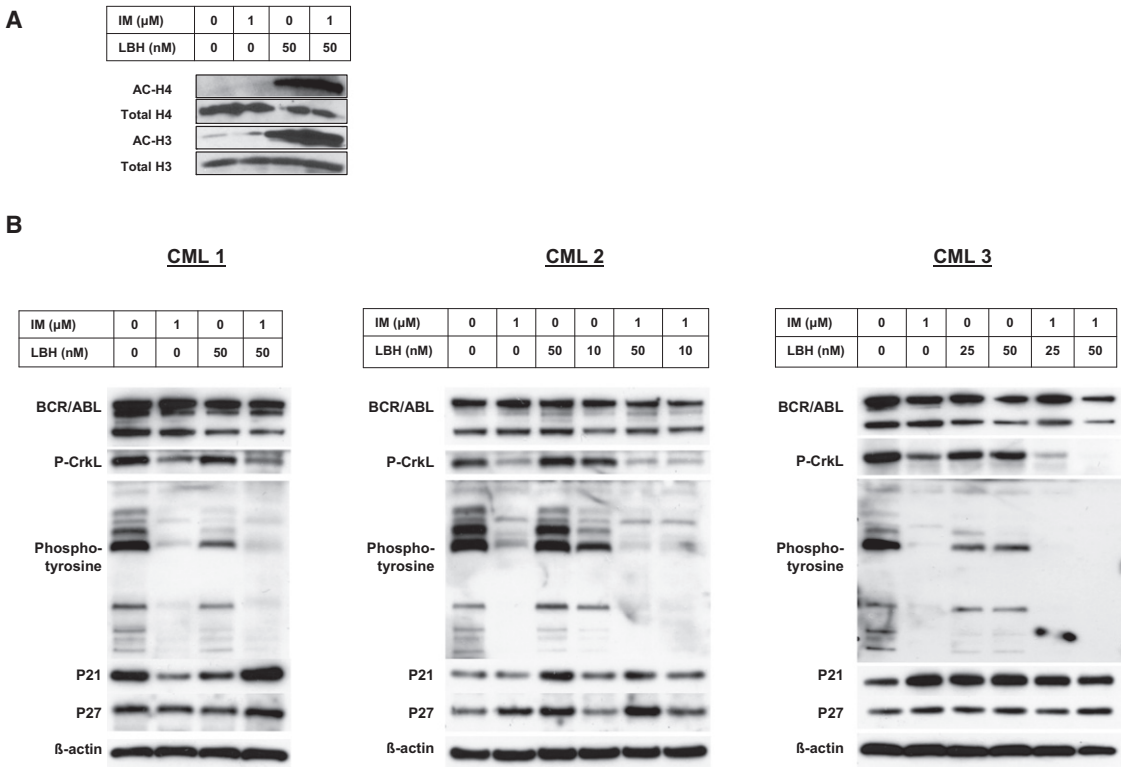


Figure 1. Effect of IM and LBH Treatment on HDAC Activity and BCR-ABL Expression and Activity in CML CD34$^+$ cells

CD34$^+$ cells from newly diagnosed patients with CML were cultured in the presence of LBH and IM for 24 hr.

(A) Histone proteins were extracted and analyzed by western blotting for levels of acetylated and total Histone H3 and H4.

(B) Total proteins were extracted and analyzed by western blotting for BCR-ABL, P-CrkL, phospho-tyrosine, p27 and p21, and actin. Results for three CML samples are shown. See also Figure S1.

recurrence is usually seen following cessation of drug treatment, even in patients with CML who are *BCR-ABL* negative by Q-PCR (Cortes et al., 2004; Rousselot et al., 2007). These observations suggest that "cure" of CML remains elusive following treatment with TKI alone.

The mechanisms underlying persistence of LSCs in IM-treated patients with CML are not well understood. BCR-ABL kinase domain mutations associated with IM resistance may be seen in some patients with CML in CCR, but are not consistently found (Chu et al., 2005). Although reduced drug uptake or increased efflux together with high levels of BCR-ABL expression in primitive progenitors could theoretically contribute to IM resistance, previous studies have found adequate drug levels and effective inhibition of BCR-ABL activity in CML progenitors following IM treatment (Chu et al., 2004; Copland et al., 2006; Jordanides et al., 2006). Our studies show that IM effectively inhibits proliferation of CML primitive progenitors but only modestly increases progenitor cell apoptosis (Graham et al., 2002; Holtz et al., 2002). Growth factor (GF) or other microenvironmental signals may preserve viability of CML cells despite BCR-ABL kinase inhibition by IM (Chu et al., 2004). Importantly, IM-induced apoptosis is restricted to dividing CML progenitors, whereas nondividing CML progenitors are especially insensitive to IM-induced apoptosis (Holtz et al., 2005; Jorgensen et al., 2005). The relative insensitivity of nondividing CML progenitors may contribute to the persistence of BCR-ABL$^+$ progenitors in patients achieving remission on IM therapy. Similar results have been obtained with more potent BCR-ABL TKIs, including dasatinib, nilotinib, and bosutinib (Copland et al., 2006; Jorgensen et al., 2007; Konig et al., 2008a; Konig et al., 2008b). These results suggest that BCR-ABL-independent mechanisms contribute to survival of primitive CML cells after TKI treatment and indicate the need to identify additional strategies to eliminate CML LSCs.

Histone deacetylase inhibitors (HDACis) are a class of agents that have shown promise as a therapy for several cancers (Marks et al., 2004). HDACis can modulate gene expression through increased histone lysine acetylation. Anticancer effects may also be related to modulation of the acetylation status of nonhistone proteins (Bolden et al., 2006). In contrast to most other proapoptotic agents that preferentially target dividing cells, HDACis have been shown to induce apoptosis in nonproliferating cancer cell lines, which may have important implications for elimination of quiescent primitive LSCs (Burgess et al., 2004). Treatment with the hydroxamic acid analog pan-HDACi SAHA, LAQ824 (LAQ), or LBH589 (LBH), alone and in combination with TKI, has been reported to induce apoptosis in CML cell lines and BC CML cells (Fiskus et al., 2006a; Fiskus et al., 2006b; Nimmanapalli et al., 2003a; Nimmanapalli et al., 2003b). However, BC CML cells may originate from a more mature progenitor population, rather than a stem cell, and differ markedly in behavior and therapeutic response from CP CML cells

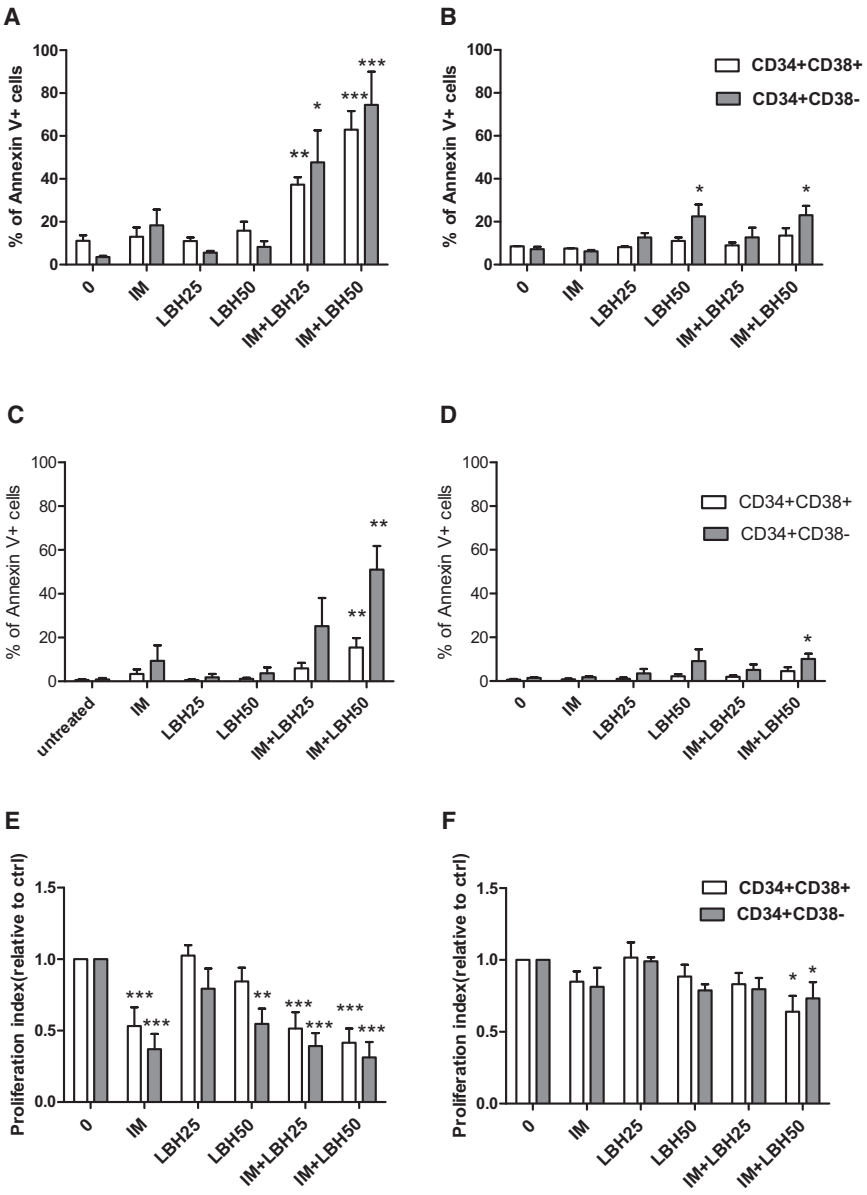


Figure 2. Apoptosis and Proliferation of CML and CB CD34$^+$CD38$^-$ Primitive Progenitors and CD34$^+$CD38$^+$ Committed Progenitors Following IM and LBH Treatment

CD34$^+$ cells from newly diagnosed patients with CML (n = 3) and CB (n = 3) were labeled with CFSE and CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ populations selected by flow cytometry sorting. Cells were exposed to LBH (25 nM or 50 nM), with or without IM (1 μM) for 96 hr. Apoptosis was analyzed by flow cytometry as the percentage of cells labeled by Annexin V-PE. Results for CML (A) and normal (B) cells are shown. Apoptosis of undivided cells for samples cotreated with LBH and 1 μM imatinib are shown for CML CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells in (C) and for normal cells in (D). Cell divisions were analyzed by flow cytometry based on reduction in CFSE intensity, and a proliferation index was determined using ModFit software. Proliferation indices were normalized to untreated controls. Results for CML (E) and normal (F) cells are shown. Results shown represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with untreated cells. See also Figure S2.

(Calabretta and Perrotti, 2004; Jamieson et al., 2004). The effect of HDACis on primitive LSCs from CP patients with CML has not been characterized. It is not known whether HDACis are capable of inducing apoptosis in quiescent stem and progenitor cells that resist elimination by BCR-ABL TKI. Here we investigated the effects of the HDACi, LAQ, and LBH, alone and in combination with IM, on primitive CML leukemia stem and progenitor cells.

RESULTS

Effect of IM and LBH Treatment on HDAC Activity and BCR-ABL Expression and Activity in CML CD34$^+$ Cells

We investigated the effects of the HDACi, LBH, and LAQ, alone and in combination with IM, on CP CML leukemia stem and progenitor cells. We confirmed that exposure to HDACis enhanced levels of acetylated histones in CML CD34$^+$ cells by western blotting of chromatin extracts. Acetylated histone H3 and H4 levels were significantly increased in cells treated with LBH (Figure 1A) or LAQ (Figures S1A–S1C available online). Histone acetylation was not altered after treatment with IM alone. Treatment with HDACis has been reported to reduce BCR-ABL expression in cell lines and BC CML cells (Fiskus et al., 2006a; Nimmanapalli et al., 2003a). We observed moderate reduction in BCR-ABL protein levels in CP CML CD34$^+$ cells after treatment with LBH (Figure 1B). Treatment with IM resulted in reduced tyrosine phosphorylation of the BCR-ABL substrate CrkL and reduced overall tyrosine phosphorylation in CML CD34$^+$ cells, confirming effective inhibition of BCR-ABL kinase activity (Figure 1B). LBH treatment also resulted in modest reduction in tyrosine phosphorylation in CML CD34$^+$ cells compared with controls, and in further reduction in tyrosine phosphorylation in combination with IM (Figure 1B). The cell cycle regulatory genes p21 and p27 are known targets of HDACi treatment. In our studies, p21 and p27 levels were observed to be increased in CML CD34$^+$ cells after LBH treatment and further increased with the combination of LBH and IM (Figure 1B).

Effects of IM and LBH Treatment on CML Progenitor Apoptosis, Proliferation, and Colony Growth

To determine the effect of LBH on apoptosis and proliferation of CML progenitor cells, we labeled CML CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), cultured them for 96 hr with LBH, IM, or the combination, and subsequently labeled them with Annexin-PE and analyzed them by flow cytometry (Figure S2A).

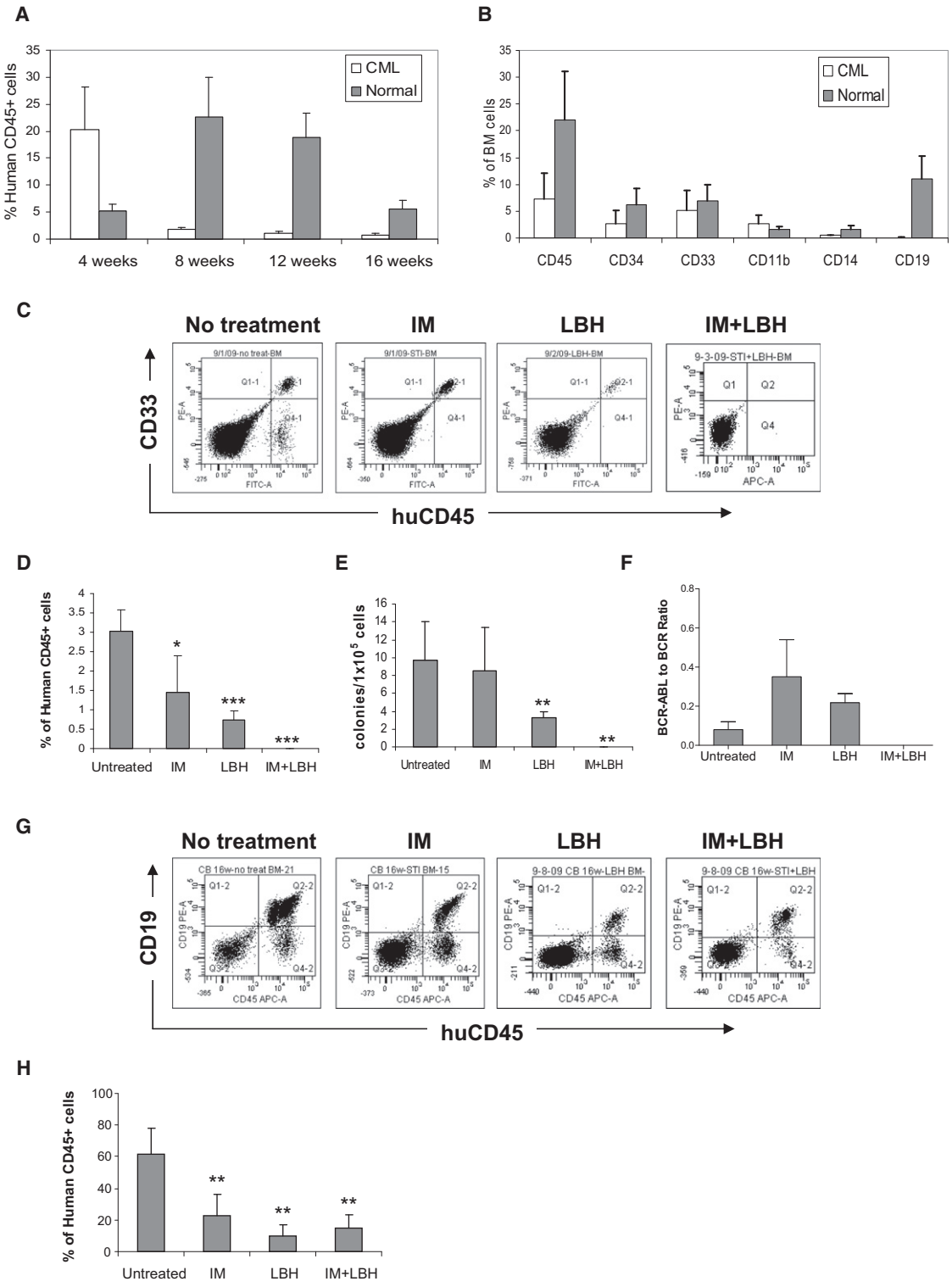


Figure 3. Combination of LBH and IM Eliminates CML Cells Capable of Long-Term Multilineage Engraftment in Immunodeficient Mice

$CD34^+$ cells from CML patients (1×10^6 to 2×10^6 cells per mouse) and normal BM (1×10^5 to 8×10^5 cells per mouse) were injected into sublethally irradiated (300 cGy) NSG mice.

(A) Human cell engraftment measured in peripheral blood samples taken from mice (n = 9 for CML; n = 7 for normal) at 4 week intervals by flow cytometric assessment of human $CD45^+$ cell engraftment is shown.

(B) BM cells were obtained from femurs of mice 16 weeks after transplantation (n = 6 for CML; n = 7 for normal) and specific human cell subsets were detected by staining with antibodies to human CD34, CD33, CD11b, and CD19. CML $CD34^+$ cells (2×10^6 cells per mouse, 8 mice per condition) and normal BM $CD34^+$ cells

Assessment of CFSE fluorescence allowed analysis of cell division since fluorescence is reduced by half in successive cell generations. We observed significantly increased apoptosis of CML $CD34^+CD38^-$ primitive and $CD34^+CD38^+$ committed progenitors following treatment with the combination of IM and LBH, but not with IM or LBH alone (Figure 2A). Treatment with 50 nM LBH with or without IM resulted in modest increase in apoptosis of normal primitive progenitors (Figure 2B). The IM and LBH combination induced significantly more apoptosis in CML compared with cord blood (CB) progenitors ($CD34^+CD38^-$ cells IM plus LBH [25 nM], $p < 0.05$; IM plus LBH [50 nM], $p < 0.001$). Similar results were observed for LAQ (Figures S2B–S2E). Reduced viability of CML $CD34^+CD38^-$ and $CD34^+CD38^+$ cells treated with the combination of IM and LBH compared with IM or LBH alone was also observed after trypan blue labeling (Figure S2P), on evaluation of cell morphology (Figure S2Q), and by Caspase 3 labeling (Figure S2R).

Previous studies have shown that primitive quiescent CML $CD34^+$ cells are especially resistant to IM-induced apoptosis (Graham et al., 2002; Holtz et al., 2005; Holyoake et al., 1999). We observed that treatment with LBH or LAQ combined with IM resulted in increased apoptosis of undivided CML $CD34^+$ cells, with CFSE fluorescence equivalent to the parent generation, compared to IM alone (Figure 2C and Figure S2F). Although LBH and LAQ treatment also increased apoptosis in undivided normal progenitor cells compared with IM alone (Figure 2D and Figure S2G), the increase in apoptosis was significantly less than in undivided CML progenitor cells ($CD34^+CD38^-$ cells IM plus LBH [25 nM], $p < 0.01$; IM plus LBH [50 nM], $p < 0.01$). In conclusion, the combination of IM and LBH effectively induces apoptosis in CML primitive and committed progenitors, and to a significantly greater extent than in normal progenitors.

Treatment of CML $CD34^+CD38^-$ primitive and $CD34^+CD38^+$ committed progenitors with IM or the combination of IM and LBH resulted in significant inhibition of proliferation as measured by reduction in CFSE fluorescence, whereas only modest reduction in proliferation of CML $CD34^+CD38^-$ cells was seen with LBH589 alone (Figure 2E). Treatment with LBH alone did not inhibit normal progenitor proliferation, whereas the combination of LBH and IM also resulted in modest inhibition of CB progenitor proliferation. Significantly greater proliferation inhibition of CML compared with CB $CD34^+CD38^-$ cells was seen for IM and IM combined with LBH 50 nM ($p < 0.05$) (Figure 2F). Similar results were seen with LAQ (Figures S2H–S2K).

The effect of LAQ on CML and normal committed progenitor frequency was assessed in methylcellulose progenitor assays (Figures S2L–S2O). Significant reduction of CML CFC frequency was seen with IM and at the highest LAQ concentration (100 nM; $p < 0.001$). LAQ combined with IM resulted in enhanced inhibition of CML CFC numbers compared with LAQ alone or IM alone ($p < 0.05$). High concentration of LAQ also resulted in reduction in normal CFC growth, which was not enhanced by combination of IM with LAQ.

Effect of IM and LBH on CML Cells Capable of Engraftment in Immunodeficient Mice

The immunodeficient nonobese diabetic/severe combined immune deficiency (NOD.CB17-*Prkdc*^{scid} or NOD/SCID) mouse model is widely used to assay primitive human hematopoietic stem cells with in vivo engraftment capacity (SCID-repopulating cells or SRC) (Larochelle et al., 1996).

NOD/SCID interleukin-2 receptor-γ chain (IL2Rγ)-deficient (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/Sz or NSG) mice support superior engraftment of human hematopoietic cells compared with NOD/SCID mice (Shultz et al., 2005). We determined that larger numbers of CML $CD34^+$ cells (1×10^6 to 2×10^6) were required to establish engraftment in NSG mice compared to $CD34^+$ cells from normal bone marrow (BM) (1×10^5 to 8×10^5). Interestingly CML LSCs demonstrated enhanced short-term compared with long-term engraftment and increased myeloid skewing compared with normal HSCs (Figures 3A and 3B).

We tested the effect of IM (1 μM), LBH (50 nM), or LBH in combination with IM (1 μM) on CML and normal cells capable of engraftment in NSG mice at 16 weeks. We observed reduced engraftment of CML $CD34^+$ cells treated with IM alone ($p < 0.05$) and LBH alone ($p < 0.001$) compared to untreated controls. However, treatment with the combination of IM and LBH resulted in further inhibition of engraftment of CML $CD34^+$ cells compared with no treatment ($p < 0.001$), IM alone ($p < 0.05$), and LBH alone ($p < 0.05$) and indeed abrogated CML cell engraftment in mice (Figures 3C and 3D). Engraftment of both myeloid and lymphoid cells was eliminated (not shown). Human CFC were also eliminated from the marrow of mice receiving cells treated with LBH combined with IM ($p < 0.01$, $p < 0.05$, and $p < 0.01$ compared with no treatment, IM alone, and LBH alone, respectively) (Figure 3E). FISH analysis showed that 86% of human cells from controls engrafted in mice were *BCR-ABL*$^+$ confirming that engraftment is truly CML in origin. Sufficient numbers of cells were not available to perform FISH analysis after combination treatment. Q-PCR analysis confirmed that *BCR-ABL*$^+$ LSCs contributed to engraftment of human cells. *BCR-ABL* and *BCR* signal were not detectable in mice receiving cells treated with the combination, confirming abrogation of engraftment of human CML cells (Figure 3F). CB $CD34^+$ cells showed significantly reduced engraftment after treatment with IM alone, LBH alone, or the combination of IM and LBH compared with no treatment ($p < 0.001$ each). Treatment with LBH and IM combination did not result in significantly enhanced inhibition of engraftment

(1×10^5 cells per mouse, seven mice per condition) were cultured for 96 hr in the absence of drug (control), with IM (1 μM) alone, LBH (50 nM) alone, or IM in combination with LBH and then transplanted into NSG mice. Mice were euthanized after 16 weeks, and PB, BM, and spleen cells were analyzed by flow cytometry.

(C and G) Representative results of engraftment in BM for CML are shown in (C) and for CB in (G).

(D and H) Combined results for engraftment of cells in BM for CML are shown in (D) and for CB in (H). Results shown represent the mean ± SEM for multiple samples. Human $CD45^+$ cells were enriched from BM of mice engrafted with CML $CD34^+$ cells with immunomagnetic column selection. The percentage of human $CD45^+$ cells present in column-selected cells were as follows: untreated, 43.9% ± 2.6%; IM, 20.9% ± 6.3%; LBH, 5.5% ± 1.5%; and IM plus LBH, 0.

(E) $CD45^+$ selected cells (1×10^5) were plated in CFC assay with human specific growth factors.

(F) *BCR-ABL* mRNA levels in $CD45^+$ selected cells were measured by Q-PCR. Results shown represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with untreated cells. See also Figure S3.

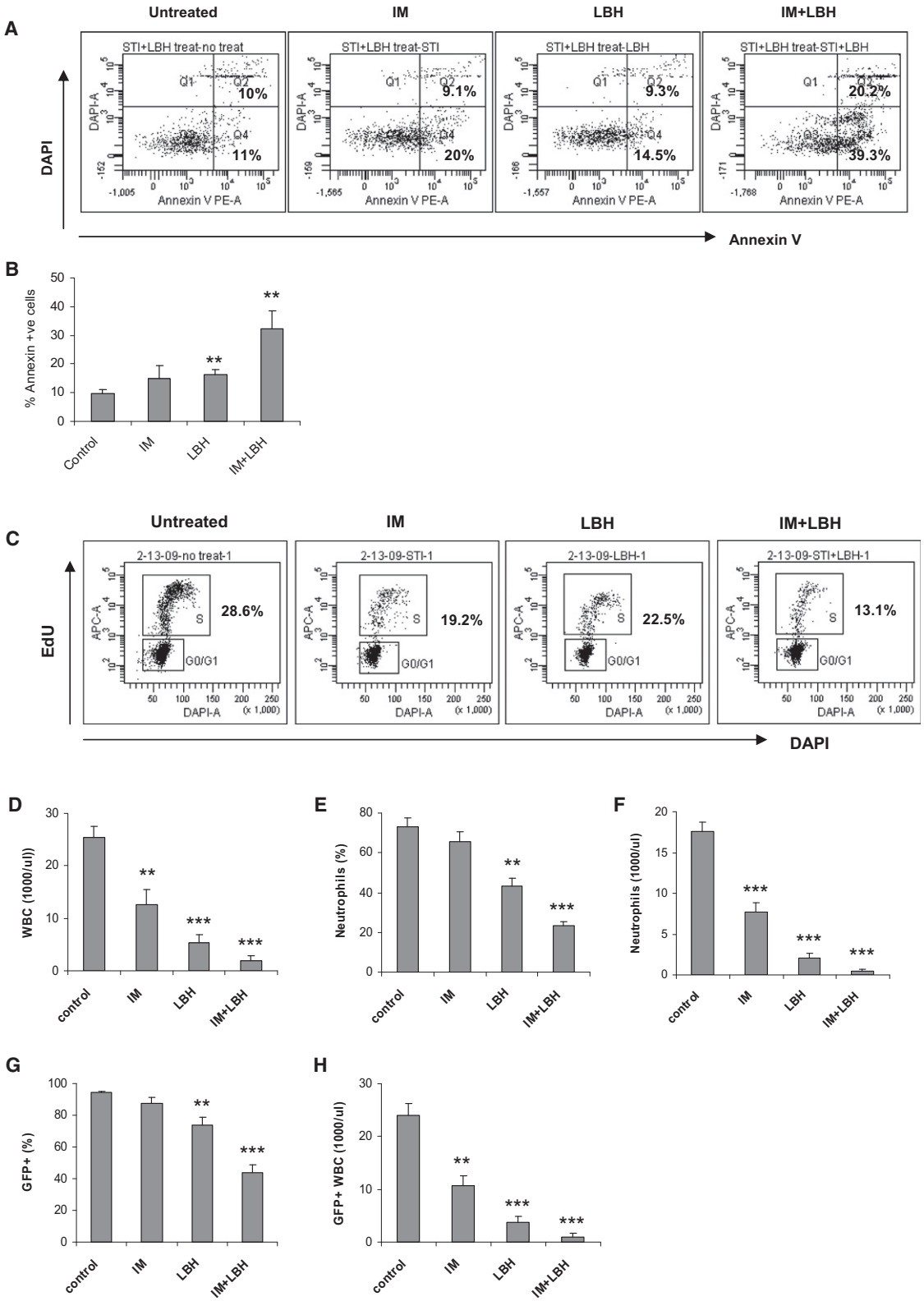


Figure 4. In Vivo Administration of LBH and IM Inhibits Myeloproliferation and Induces Apoptosis in LSCs in Transgenic BCR-ABL–Expressing Mice

BCR-ABL expression was induced in Scl-tTa-BCR-ABL/GFP mice by tetracycline withdrawal. BM cells were obtained 4 weeks after induction, and GFP-expressing cells selected using flow cytometry were transplanted into wild-type FVB/N recipient mice irradiated at 900 cGy (10^6 cells /mouse). Treatment with IM

compared to IM alone ($p = 0.37$) or LBH alone ($p = 0.43$) (Figures 3G and 3H). Significantly less inhibition of normal cell engraftment was seen after LBH and IM treatment compared to CML cell engraftment, which was completely eliminated ($p = 0.01$). Similar results were obtained when evaluating the effect of LAQ treatment on engraftment of CML and normal cells in NSG mice at 6 weeks (Figures S3A–S3E). These results show that HDACis in combination with IM effectively target primitive CML cells capable of multilineage engraftment that resist elimination following treatment with IM alone.

Effect of In Vivo Administration of LBH and IM on Leukemia Stem Cells in Transgenic BCR-ABL-Expressing Mice

The low levels of long-term engraftment of CML LSCs in the xenogeneic transplant model limit its use for in vivo drug treatment studies. We therefore utilized a transgenic Scl-tTa-BCR-ABL mouse model to investigate the effect of HDACi treatment on CML stem cells in vivo (Huettner et al., 2003). Withdrawal of tetracycline results in reversible induction of BCR-ABL expression and induction of a CML-like myeloproliferative disorder characterized by neutrophilic leukocytosis and splenomegaly. Transplantation of leukemic BM cells to wild-type recipients consistently resulted in development of a myeloproliferative disorder 3–4 weeks after transplantation. The transplantation approach allows generation of a large cohort of mice with similar time of onset of leukemia, providing a robust and consistent CML model suitable for preclinical studies of therapeutic interventions against CML stem cells in the setting of the in vivo host microenvironment.

Scl-tTa-BCR-ABL mice were crossed with transgenic GFP-expressing mice to facilitate identification of donor cells. BM cells were obtained from Scl-tTa-BCR-ABL/GFP mice 4 weeks after induction of BCR-ABL expression by tetracycline withdrawal. GFP-expressing cells were selected using flow cytometry and transplanted into wild-type FVB/N recipient mice irradiated at 900 cGy. Recipient mice developed leukocytosis and neutrophilia 4 weeks after transplantation. Mice were then treated for 4 weeks with IM (200 mg/kg body weight daily by gavage), LBH (30 mg/kg body weight intraperitoneally 3 days per week on Monday, Wednesday, and Friday), LBH in combination with IM, or vehicle alone (controls). Mice treated with the IM and LBH combination demonstrated significantly increased apoptosis of Lin-Sca-1$^+$Kit$^+$ (LSK) stem cells compared with vehicle or either drug alone ($p < 0.005$ Figures 4A and 4B). In vivo EdU labeling studies showed reduction in the percentage of LSK cells in S-phase in mice treated with the combination of IM and LBH and to a lesser extent with IM (Figure 4C). Levels of white blood cells (WBCs), neutrophils, and GFP$^+$ cells (representing BCR-ABL expressing cells) were elevated in controls but were reduced to normal levels in mice receiving the different treatments (Figures 4D–4H). LBH combined with IM resulted in greater reduction in WBC, neutrophils, and GFP$^+$ cells than single agent LBH or IM. Control mice became moribund, and three of ten mice died between 3 and 4 weeks after start of treatment (7–8 weeks after transplantation), whereas the mice receiving the different treatments remained well. Mice were euthanized at 4 weeks and BM and spleen cells were analyzed by flow cytometry. A profound reduction of GFP$^+$ WBCs (Figure 5B), myeloid cells (Gr-1$^+$Mac-1$^+$, Figure 5C), granulocyte-macrophage progenitors (GMP, Lin-Kit$^+$Sca-1-CD34$^+$FcγRII/III^{hi}, Figure 5D), common myeloid progenitors (CMP, Lin-Kit$^+$ Sca-1-CD34$^+$FcγRII/III^{lo}, Figure 5E), and stem cells (LSK cells, Figures 5F and 5G) was observed in the BM of mice treated with IM combined with LBH compared with IM or LBH alone. LBH as a single agent also reduced GFP$^+$ WBCs and progenitors but to a significantly lesser extent than the combination. A marked reduction of total GFP$^+$ WBCs (Figure 5H), immature myeloid cells (Gr-1$^+$Mac-1$^+$) (Figure 5I), GMP (Figure 5J), CMP (Figure 5K), and LSK cells (Figures 5L and 5M) was also seen in spleens of mice treated with IM in combination with LBH. In contrast to effects on BM cells, LBH as a single agent was ineffective in reducing numbers of GFP$^+$ progenitors in the spleen. Treatment of wild-type FVBN mice with IM, LBH, or the combination for 4 weeks did not cause clinical symptoms or affect survival. There was a trend toward less weight gain in treated mice compared with controls, although it was not statistically significant (Figure S4A). Treatment with LBH or LBH combined with IM caused moderate suppression of WBC and red blood cell (RBC) counts in peripheral blood and suppression of GMP, MEP, and LSK cells in BM from wild-type mice (Figures S4B–S4G). Therefore, LBH with or without IM results in moderate in vivo toxicity to normal hematopoietic cells, although considerably less than the toxicity to leukemia cells.

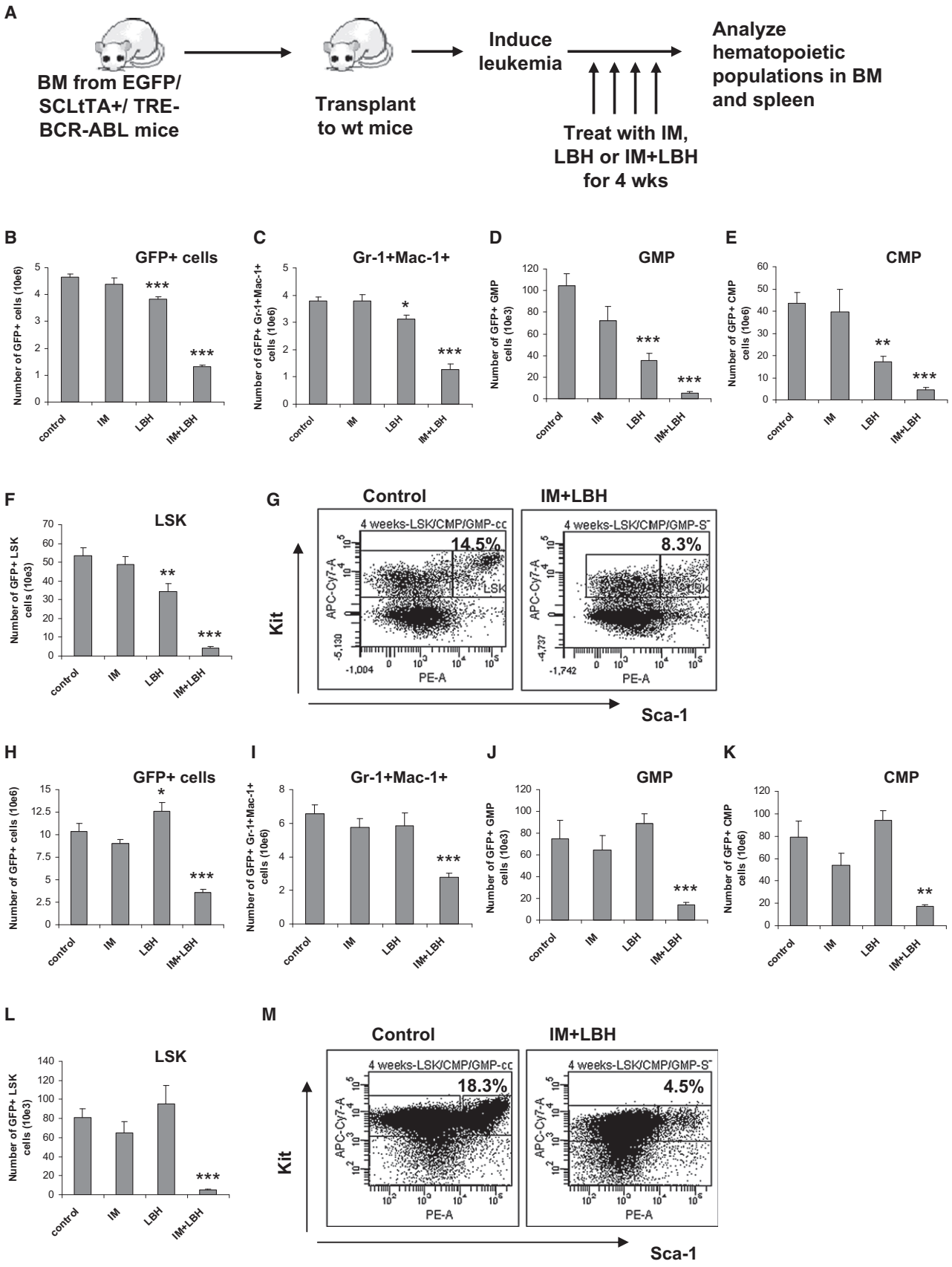
We conducted survival studies on mice after 4 weeks exposure to treatment (Figure 6A). Control mice that survived the 4 weeks treatment period all died within 90 days after discontinuing treatment (Figure 6B). Mice treated with the combination of IM and LBH showed markedly improved survival, and showed normal WBC counts at 8 weeks after discontinuation of treatment with only small number of residual GFP$^+$ WBCs (Figure 6C and 6D). To accurately quantify the loss of CML stem cells, we transplanted BM cells from leukemic mice treated with IM, LBH, or the combination for 4 weeks in limiting dilutions to secondary mice (Figure 6E). Treatment with the combination of LBH and IM resulted in abrogation of engraftment of GFP$^+$ leukemia cells in secondary mice measured at 8 and 16 weeks after transplantation (Figure 6F–6H). In contrast, LBH alone or

(200 mg/kg daily by gavage), LBH (30 mg/kg body weight intraperitoneally 3 days per week on Monday, Wednesday, and Friday), or LBH in combination with IM or vehicle alone (controls) was initiated 4 weeks after transplantation and continued for 4 weeks. Apoptosis in BM LSK cells was evaluated 5 days after start of treatment by Annexin-V and DAPI labeling.

(A and B) Representative results are shown in (A), and compiled results from three experiments are shown in (B).

(C) For evaluation of stem cell cycling, mice were injected intraperitoneally with EdU and killed 2 hr later. The percentage of stem cells in S-phase was determined on the basis of EdU incorporation in BM LSK cells measured by flow cytometry as described in Experimental Procedures. Representative results from one of two such experiments are shown.

(D–H) PB total WBC counts (D), neutrophil percentage and absolute neutrophil counts (E and F), and GFP$^+$ WBC percentage and absolute counts (G and H) were measured 4 weeks after start of treatment. Results represent the mean ± SEM for five to six mice per treatment. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with no treatment.


A
BM from EGFP/ SCLtTA+/ TRE-BCR-ABL mice
Transplant to wt mice
Induce leukemia
Treat with IM, LBH or IM+LBH for 4 wks
Analyze hematopoietic populations in BM and spleen
B
GFP+ cells
Number of GFP+ cells (10e6)
C
Gr-1+Mac-1+
Number of GFP+ Gr-1+Mac-1+ cells (10e6)
D
GMP
Number of GFP+ GMP cells (10e3)
E
CMP
Number of GFP+ CMP cells (10e6)
control
IM
LBH
IM+LBH
F
LSK
Number of GFP+ LSK cells (10e3)
G
Control
IM+LBH
4 weeks-LSK/CMP/GMP-cc
4 weeks-LSK/CMP/GMP-S
14.5%
8.3%
LSK
APC-Cy7-A
PE-A
Kit
Sca-1
H
GFP+ cells
I
Gr-1+Mac-1+
J
GMP
K
CMP
L
LSK
M
Control
IM+LBH
18.3%
4.5%

IM alone did not significantly affect levels of GFP$^+$ cells in secondary recipients (Figure 6F) or the frequency of LSCs quantified on limiting dilution analysis (Figures 6G and 6H). These results clearly demonstrate that the LBH and IM combination is capable of markedly reducing the number of LSCs capable of engraftment in secondary recipients and causing disease relapse.

Effect of IM and LBH on Gene Expression in CML Progenitors

To further investigate potential mechanisms underlying CML LSC targeting, we conducted gene expression analysis of CML CD34$^+$CD38$^-$ cells exposed to IM, LBH, and the combination of IM and LBH. We focused our analysis on genes whose expression was significantly affected by the combination of IM and HDACis, compared with IM and HDACis alone, which we refer to as the ''interaction'' of IM and LBH (Figure 7A). The gene expression programs represented by these genes were identified with Gene Set Enrichment Analysis (GSEA). IM and LBH combination resulted in increased inhibition of HDACi-downregulated gene sets compared to LBH alone, suggesting that cotreatment with IM could potentiate HDACi-induced gene expression alterations. We observed reduced expression of gene sets related to the HSC state; the HOX-, MYC-, and WNT-related pathways; cell-cycle regulation; protein translation through the mTOR-EIF4 pathway; cellular stress response and cell survival; and increased expression of G protein-coupled receptor genes (Figure 7B and Table 1). We also observed reduced expression of genes with transcription factor (TF) binding motifs for E2F, which has important roles in regulating cell-cycle progression and apoptosis (Chen et al., 2009); Ying-Yang 1, which directs histone acetylation changes at promoters (Gordon et al., 2006); GABP/NRF2 and NRF1, which regulate stress response (Mathers et al., 2004); and MYC-MAX, which regulate HSC proliferation and maintenance (Laurenti et al., 2008) (Table 1). These results suggest possible molecular mechanisms that may contribute to the effects of the HDACi and IM combination (Figure 7C).

DISCUSSION

Several leukemias, including CML, are propagated by small populations of LSCs, eradication of which may be required to achieve long-term remission and cure. Populations of cancer stem cells have also been identified in several solid tumors (Clarke et al., 2006). Treatment with BCR-ABL TKI, including IM, nilotinib, and dasatinib, reverses the proliferative advantage of CML progenitors, resulting in remission induction, but does not eliminate LSCs, which continue to be detectable in patients despite prolonged IM treatment. Persistent LSCs represent a reservoir of disease and potential source of relapse. Patients with CML have a high likelihood of disease relapse on discontinuation of treatment, and it is currently recommended that patients should be treated with IM indefinitely to prevent relapse. Concerns regarding the lack of disease cure with IM alone, the potential risk of adverse effects with long-term treatment, issues of noncompliance, and the high financial burden associated with drug treatment provide a strong impetus to develop strategies to target residual LSCs.

In the current study, we found that treatment with HDACis in combination with IM was significantly more effective in inducing apoptosis in CP CML progenitor cells compared with IM alone. The combination of HDACis and IM induced apoptosis in quiescent CML progenitor cells that are highly resistant to elimination following treatment with IM alone. HDACis in combination with IM also inhibited CML progenitor proliferation and CML committed progenitor growth in colony assays. In contrast to reports that HDACis could induce apoptosis in CML cell lines and BC CML cells (Fiskus et al., 2006a; Fiskus et al., 2006b; Nimmanapalli et al., 2003a; Nimmanapalli et al., 2003b), we found that HDACis by themselves had minimal effect on apoptosis of CP CML cells. CML progenitor proliferation and CFC frequency were reduced only at the highest HDACi concentrations. Therefore, targeting of primitive CP CML cells by HDACis is potentiated by concomitant inhibition of BCR-ABL tyrosine kinase activity. Importantly, treatment with the combination of HDACis and IM markedly depleted CML LSCs capable of long-term, multilineage engraftment in immunodeficient mice. Furthermore, administration of the HDACis and IM in combination resulted in profound depletion of LSCs with secondary repopulating capacity in the in vivo setting in a transgenic BCR-ABL mouse model of CML, and prevented leukemia relapse after discontinuation of treatment. Therefore, our data consistently show that the combination of IM with an HDAC inhibitor effectively targets primitive CML LSCs and is superior to either agent alone. On the other hand, this combination may be less suitable for treating patients harboring IM resistance-conferring *BCR-ABL* mutations, since the mutant kinase would be inhibited poorly by IM, thus eliminating its required contribution to the effect of the combination.

The effects of HDACis are complex and involve multiple genes and pathways. HDACi-induced reduction in BCR-ABL protein expression and kinase activity could potentiate the effects of IM on CML LSCs. We also observed that combination with IM enhanced HDACis-induced gene expression changes, compared with HDACis alone. Although the mechanisms underlying this effect are still unknown, novel nuclear functions of

Figure 5. LBH and IM Administration Profoundly Depletes Leukemia Stem and Progenitor Cells in the BM and Spleen of Transgenic BCR-ABL–Expressing Mice

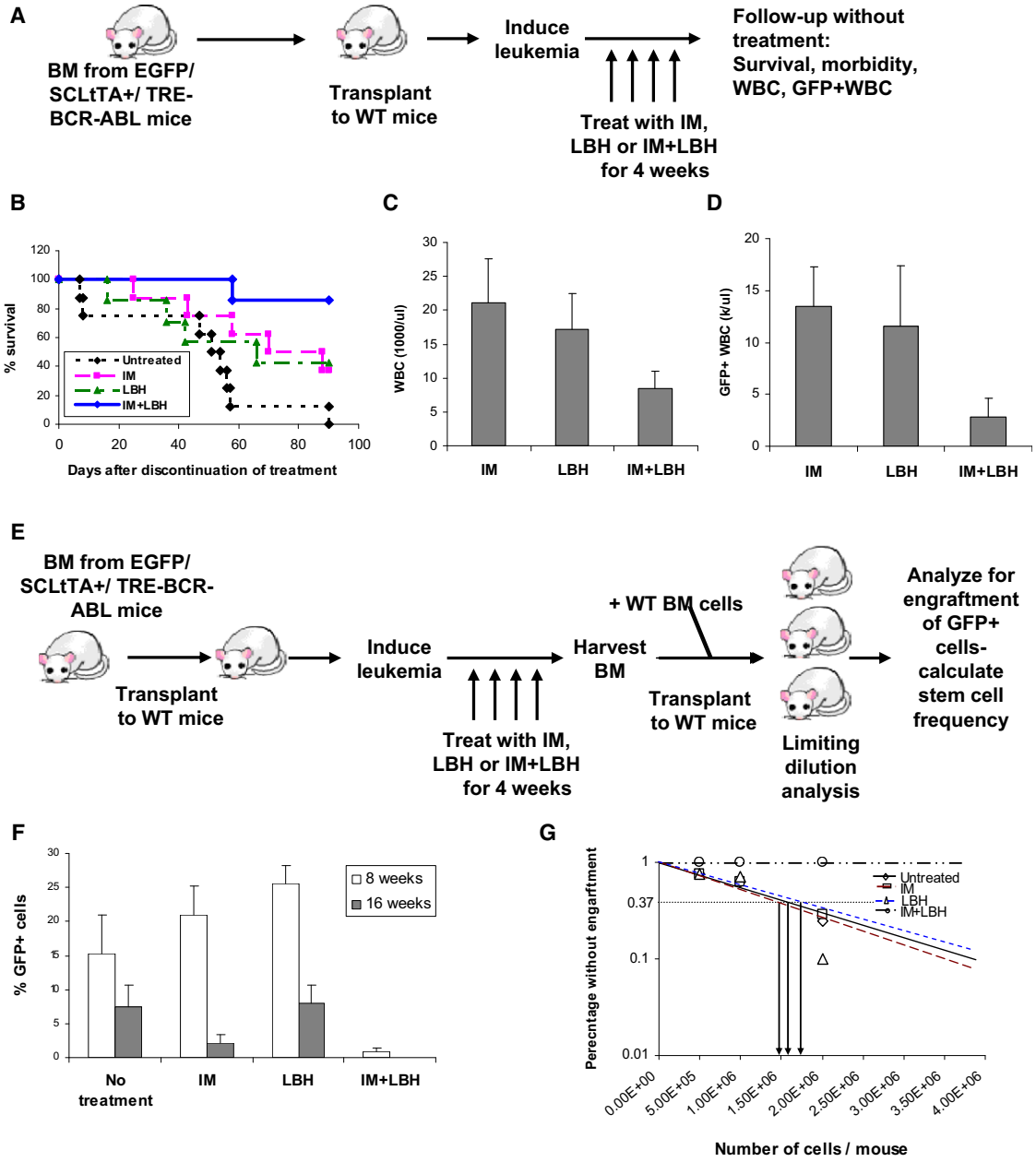
(A) Mice from the cohort described in Figure 4 were euthanized after 4 weeks of treatment with IM, LBH, IM combined with LBH, or vehicle alone and BM, and spleen cells were obtained). GFP-expressing hematopoietic populations were analyzed by flow cytometry.

(B–F) Results for the following GFP$^+$ populations in the BM (n = 6 mice per treatment) are shown: total GFP$^+$ WBCs (B), immature myeloid cells (Gr-1$^+$Mac-1$^+$) (C), GMP (D), CMP (E), and LSCs (LSK cells) (F).

(G) Representative flow cytometry plot for BM LSK cells is shown.

(H–L) Results for GFP$^+$ populations in the spleens (n = 5 mice per treatment) are as follows: total GFP$^+$ WBCs (H), Gr-1$^+$Mac-1$^+$ cells (I), GMP (J), CMP (K), and LSK cells (L).

(M) A representative flow cytometry plot for splenic LSK cells is shown. Results represent the mean ± SEM for multiple samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with no treatment. See also Figure S4.



H

Cell dose	Engrafted/Tested			
	No treatment	IM	LBH	IM+LBH
2,000,000	6/8	5/7	4/4	0/6
1,000,000	3/8	3/8	2/7	0/8
500,000	2/8	2/8	2/8	0/8
LT-HSC frequency	5.89E-07	5.57E-07	6.38E-07	0

Figure 6. Transplantation of BM from IM and LBH Treated Mice into Secondary Recipients

(A) BCR-ABL expression was induced in GFP$^+$/SCLtTA/BCR-ABL mice, and BM cells were obtained from mice 4 weeks after induction. GFP-expressing cells were selected and transplanted into wild-type FVB/N recipient mice (10^6 cells/mouse). Treatment with IM, LBH, LBH in combination with IM, or vehicle alone was initiated 4 weeks after transplantation and was continued for 4 weeks (eight mice per condition) as described for Figures 4 and 5.

(B) Mice were followed after discontinuation of treatment and survival was monitored for 90 days.

(C and D) The total WBC count (C) and GFP$^+$ WBC count (D) in PB of mice 8 weeks after discontinuation of treatment are shown.

(E) BCR-ABL expression was induced in GFP$^+$/SCLtTA/BCR-ABL mice, and BM cells were obtained 4 weeks after induction. GFP-expressing cells were transplanted into wild-type FVB/N recipient mice irradiated at 900 cGy (10^6 cells/mouse). Treatment with IM, LBH, and LBH in combination with IM, or vehicle alone was initiated 4 weeks after transplantation and continued for 4 weeks (five mice per condition). Mice were then euthanized, and BM cells were obtained.

cytoplasmic tyrosine kinases related to histone and transcription factor modulation have been recognized. Potential mechanisms involved in CML LSC inhibition by the IM and HDACi combination, compared with IM or HDACis alone, suggested by gene expression analyses included reduced expression of genes related to the primitive HSC state such as downregulation of HOX-, MYC-, and WNT-related genes; reduced expression of E2F-regulated genes, which may play an important role in protecting nonproliferating cells from stress-induced apoptosis (Moon et al., 2005); and increased G protein coupled receptor expression, possibly influencing microenvironmental interactions of CML progenitors (Figure 7C). These observations, although preliminary, indicate promising avenues for further investigation of molecular mechanisms underlying the effects of the HDACi and IM combination.

Our studies indicate significant activity of HDACis against normal hematopoietic progenitors with increased levels of apoptosis, inhibition of proliferation, and inhibition of CFC and SRC growth. In vivo administration of LBH was associated with moderate inhibition of normal blood cell counts and BM stem and progenitor populations and reduced weight gain. On the one hand, these inhibitory effects on normal progenitors are consistent with clinical observations of thrombocytopenia and myelosuppression in clinical trials of LBH (Bruserud et al., 2007). On the other hand, the HDACi and IM combination resulted in significantly less apoptosis of normal compared with CML progenitors and significantly less inhibition of normal SRC compared with CML SRC. In addition, in vivo administration of the combination resulted in significantly less inhibition of normal blood cell counts, and BM stem and progenitor populations, compared with near complete elimination of CML LSCs. These observations suggest a therapeutic window for HDACi and IM effects on CML LSCs compared with normal stem cells that could be exploited clinically. However, the toxicity of LBH to normal progenitors indicates a need for continued exploration of mechanisms underlying activity of the LBH and IM combination on CML LSCs to aid development of more selective, nontoxic approaches for targeting LSCs in future.

There is considerable interest in devising improved approaches to target CML LSCs. The therapeutic application of these studies will be toward using a combination of LBH and IM to achieve elimination of residual LSCs in IM-treated and responsive patients with CML. We have recently shown that a farnesyltransferase inhibitor BMS-214662 can selectively kill quiescent primitive CML progenitor cells (Copland et al., 2008). However, this agent is not being developed for clinical testing in CML. In contrast, several HDACis are currently in clinical trials for hematological malignancies as well as solid tumors (Glaser, 2007). The role of HDACis in targeting cancer stem cells has not been previously described. On the basis of our observation that LAQ and LBH combined with IM can eliminate CML LSCs, we have developed and initiated a clinical trial to determine the safety and tolerability of LBH in combination with IM in patients with CML who are in cytogenetic remission with evidence of residual *BCR-ABL*$^+$ cells. The ultimate measure of success for these studies in achieving elimination of residual LSCs will be the ability of patients to maintain long-term remission after discontinuation of IM treatment. Historically, studies in CML have greatly enhanced our understanding of chromosomal translocations and oncogenes in cancer biology and have led the way in successful application of targeted therapies. It remains to be determined whether the current studies of targeting of LSCs in CML using HDACis will have broader application to targeting of primitive, quiescent cancer-initiating cells in other leukemias and solid tumors.

EXPERIMENTAL PROCEDURES

Samples

CB samples were kindly provided by StemCyte (Arcadia, CA). Normal BM samples were obtained from donors at City of Hope National Medical Center (COHNMC). Mononuclear cells were isolated using Ficol separation. CD34$^+$ cells were then isolated using a positive magnetic bead selection protocol (StemCell Technologies, Vancouver, BC, Canada). CML samples were obtained from patients in CP who had not received prior IM treatment from the COHNMC and Glasgow University. BM samples were processed as described above. Leukopheresis samples were processed for CD34$^+$ cell selection with CliniMACS (Miltenyi Biotech, Germany). CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells were obtained by flow cytometry sorting. All patients and healthy donors signed an informed consent form. Sample acquisition was approved by the Institutional Review Boards at the COHNMC, in accordance with an assurance filed with and approved by the Department of Health and Human Services, and the North Glasgow University Hospital Division of NHS Greater Glasgow and Clyde, and met all requirements of the Declaration of Helsinki.

Cell Culture

Cells were exposed to IM, LBH, and LAQ (Novartis) during culture in Stemspan serum-free medium (StemCell Technologies), supplemented with low concentrations of growth factors (GF) similar to those present in long-term BM culture stroma-conditioned medium (200 pg/mL granulocyte-macrophage colony-stimulating factor [GM-CSF], 50 pg/mL leukemia inhibitory factor [LIF], 1 ng/mL granulocyte colony-stimulating factor [G-CSF], 200 pg/mL stem cell factor [SCF], 200 pg/mL macrophage-inflammatory protein-1α [MIP-1α], and 1 ng/mL interleukin-6 [IL-6]) at 37°C with 5% CO_2 and high humidity (Bhatia et al., 1995).

Analysis of Proliferation and Apoptosis

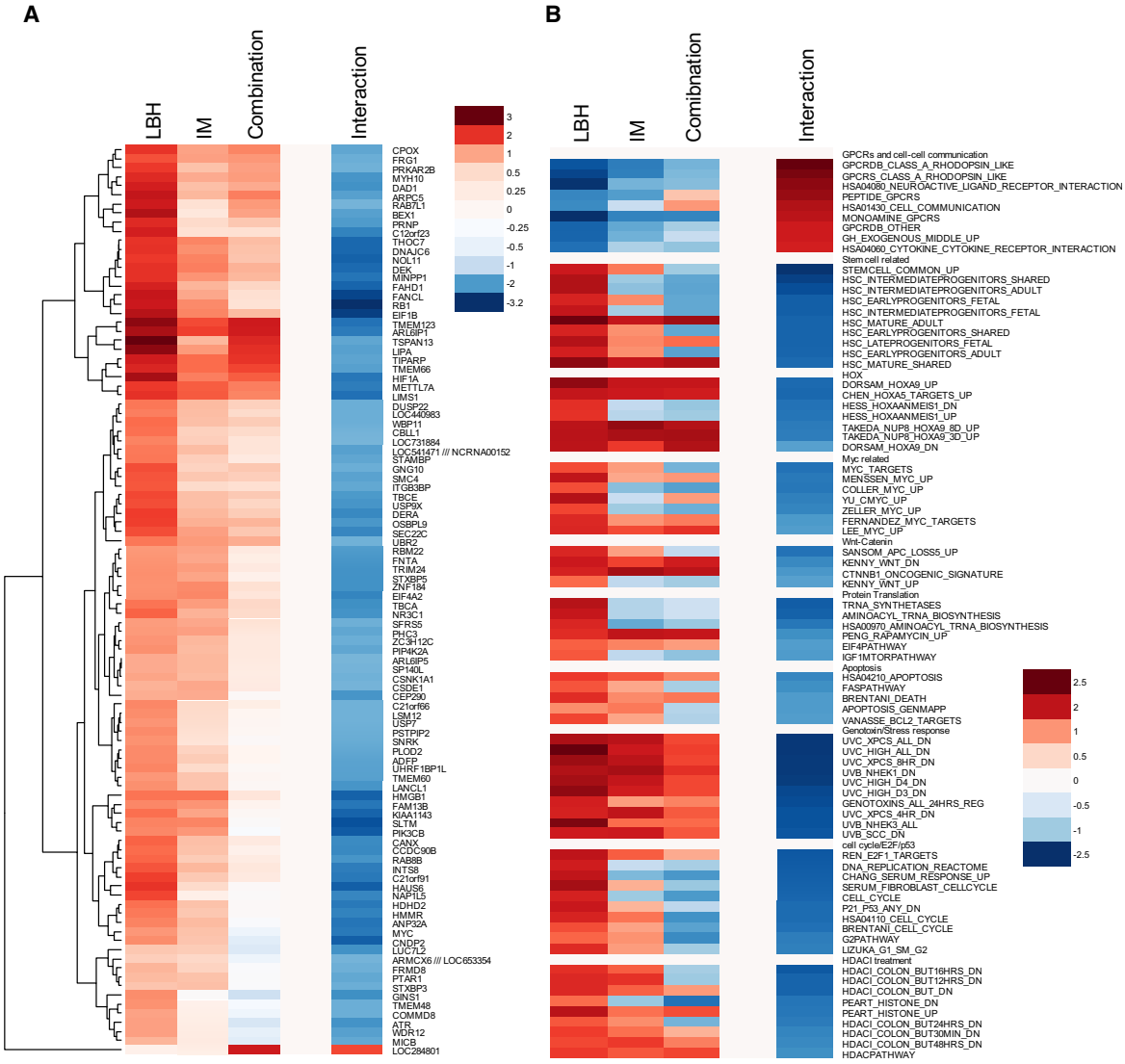
Cells were labeled with 1.25 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C, were incubated overnight in Stemspan serum-free medium supplemented with low concentrations of growth factors, as described above, for releasing unbound CFSE, were labeled with CD34-APC and CD38-PE, and were flow-cytometry sorted (MoFlo; Cytomation, Fort Collins, CO) for CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ cells with narrow and uniform CFSE labeling. Cells were then cultured as described above for 96 hr with or without IM, LBH, or LAQ, were labeled with Annexin V-PE (BD-PharMingen, San Diego, CA), and were analyzed by flow cytometry (FACSCalibur; BD) for Annexin V and CFSE fluorescence. The level of CFSE in the parent cells was determined using

The BM cells were pooled from 3–5 mice, and 2 × 10^6, 1 × 10^6, or 5 × 10^5 cells/mouse (eight mice per dose per condition) mixed with 2 × 10^5 BM cells/mouse from wild-type FVBN mice were transplanted into wild-type FVB/N recipient mice irradiated at 900 cGy. Engraftment was monitored by drawing PB every 4 weeks. The percentage of GFP$^+$ cells in PB was analyzed by flow cytometry.

(F) The mean ± SEM of GFP$^+$ WBCs at 8 and 16 weeks after transplantation are shown. A percentage of GFP$^+$ cells in PB $\geq$0.5% was considered positive for engraftment.

(G) The frequency of LSCs after treatment is shown.

(H) The fraction of mice showing evidence of engraftment at 16 weeks after secondary transplantation is shown.


A
LBH
IM
Combination
Interaction
B
LBH
IM
Comibnation
Interaction
C
LBH + IM
↑ Histone deacetylation
↓ Kinase activity
Altered transcriptional regulation
↓ YY1
↑ GPCR
↓ Myc
↓ Wnt-catenin
↓ Hox A9, Hox A5
↓ E2F
↓GABP ARNT
↓ Translation
↓ Ribosomes
↓ mTOR-EIF4
↓ HSC maintenance & self-renewal
↓ Proliferation
↓ Stress response
↑ Apoptosis

aliquots of cells fixed in 4% paraformaldehyde directly after cell sorting. ModFit software (Verity, Topsham, ME) was used for assessment of the different cell generations and proliferation index of each sample. The percentage of apoptotic cells in total, divided, and undivided cells was determined. The effect of drug treatment on cell viability was also assessed by trypan blue staining, assessment of cell morphology and Caspase 3 labeling as described in the Supplemental Experimental Procedures.

Engraftment of Human Cells in Immunodeficient Mice

CML $CD34^+$ cells (1–2 × 10^6 cells/ mouse) or CB $CD34^+$ cells (1 × 10^5 cells/ mouse) were cultured for 96 hr in the absence of drug (control), or with addition of IM (1 μM) alone, LBH (50 nM) alone, or IM (1 μM) in combination with LBH or LAQ (50 nM) in medium with low concentrations of GF. Cells were then harvested, washed, and transplanted via tail vein injection into sublethally irradiated (300 cGy) 8-week-old NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tm1Wjl} /SzJ mice (NSG mice, Jackson Laboratory, Bar Harbor, ME). Mice were euthanized after 6 or 16 weeks, and marrow contents of femurs, spleen cells, and blood cells were obtained at necropsy. For assessing human cell engraftment, cells were labeled with anti-human CD45 antibody and analyzed by flow cytometry. Specific human cell subsets were detected by staining with antibodies to human CD34, CD33, CD11b, and CD19. Human $CD45^+$ cells were selected by immunomagnetic column selection. For assessing human CFC, $CD45^+$ selected cells were placed in methylcellulose progenitor culture with human specific cytokines. For assessing engraftment of malignant BCR-ABL–expressing cells, $CD45^+$ selected cells obtained were evaluated for the BCR-ABL translocation by interphase FISH and for *BCR-ABL* mRNA levels by Q-PCR. Mouse care and experimental procedures were performed in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at COHNMC.

In Vivo Treatment of Transgenic BCR-ABL Mice

Inducible, transgenic Scl-tTa-BCR-ABL mice in the FVB/N background (Huettner et al., 2003) were crossed with transgenic GFP-expressing mice (FVB.Cg-Tg [ACTB-EGFP] B5Nagy/J, Jackson Laboratories). BM cells were obtained from Scl-tTa-BCR-ABL/GFP mice 4 weeks after induction of BCR-ABL expression by tetracycline withdrawal, and a pure population of GFP-expressing cells was selected with flow cytometry and was transplanted by tail vein injection (10^6 cells/mouse) into wild-type FVB/N recipient mice irradiated at 900 cGy. Blood samples were obtained 4 weeks after transplantation to confirm development of neutrophilic leukocytosis. Mice were treated with IM (200 mg/kg daily by gavage for 28 days), LBH (30 mg/kg body weight intraperitoneally three times a week for 28 days), LBH in combination with IM, or with vehicle alone (control). After 4 weeks of treatment, animals were euthanized, and the total marrow content of femurs and tibiae and spleen cells were obtained. The number of total nucleated cells, GFP-expressing cells, and GFP^+ myeloid, progenitor, and stem cell populations were measured by flow cytometry, as described in Supplemental Experimental Procedures. The effect of drug administration on apoptosis and cycling of stem cells in vivo was evaluated as described in the Supplemental Experimental Procedures. BM cells from a subset of treated mice were pooled and 2 × 10^6, 1 × 10^6, 5 × 10^5 cells/mouse (eight mice per dose per condition) mixed with 2 × 10^5 BM cells/mouse from wild-type FVBN mice were transplanted into wild-type FVB/N recipient mice irradiated at 900 cGy. Engraftment was monitored by drawing peripheral blood (PB) every 4 weeks. The percentage of GFP^+ cells in PB was analyzed by flow cytometry. The fraction of mice showing evidence of engraftment at 16 weeks after secondary transplantation was determined, and the frequency of LSCs was calculated using Poisson statistics. Another subset of mice was followed after discontinuation of treatment, and survival and PB counts were monitored for 90 days. Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at COHNMC.

Real-Time Q-PCR Analysis

For measuring *BCR-ABL* mRNA in cells engrafted in mice, total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Valencia, CA), and first-strand cDNA was synthesized with Superscript III first strand kit and Q-PCR analysis performed with primer and probe sequences for *BCR-ABL* (B3A2), as described elsewhere (Branford et al., 1999). *BCR* levels were measured as internal controls. The amount of *BCR-ABL* mRNA per unit input RNA was calculated on the basis of the standard curves.

Western Blotting

Cells were cultured with or without IM, LBH, and LAQ for 24 hr. For analysis of histones, cells were lysed in buffer containing 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% sodium azide; chromatin was isolated with centrifugation, and histones were extracted overnight in 0.2 N HCl. For evaluation of other proteins, cells were lysed in buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail (all from Sigma Diagnostics). Proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and were transferred to nitrocellulose membrane. Membranes were sequentially reprobed with primary and secondary antibodies. Primary antibodies included anti-Histone-H3 and anti-Histone-H4 rabbit polyclonal and anti-acetyl-Histone-H3 (Lys9/Lys14) rabbit polyclonal antibody (9715 and 9677) (Cell Signaling Technology, Danvers, MA), anti-acetyl H4 (Millipore, Billerica, MA), anti-P-CrkL (Cell Signaling Technology), anti-phosphotyrosine (Upstate), anti-P27 (c-19) and anti-P21(C-19) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin mouse monoclonal antibody (AC-15) (Sigma-Aldrich Corp., St. Louis, MO), and anti-ABL (Ab-3) (Oncogene Science, Cambridge, MA). Horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (Westgrove, PA). Antibody detection was performed with the Superfemto kit (Pierce Biotechnology, Rockford, IL). Protein levels were determined by densitometry with Image-Quant software (Amersham Pharmacia Biotech, Piscataway, NJ).

Analysis of Gene Expression Changes Induced by Combined IM and LBH Treatment

CML $CD34^+CD38^-$ cells selected with flow cytometry sorting were treated with IM (1 μM), LBH (50 nM), and the combination of IM and LBH or cultured without exposure to drugs (controls) for 24 hr (n = 3 each). Total RNA from 5000 cells was extracted with the RNeasy kit (QIAGEN), was amplified and labeled with GeneChip Two-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA), and was hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays. Microarray data analyses were performed with R (version 2.9) with genomic analysis packages from

Figure 7. Gene Expression Changes Induced by Combined IM and LBH Treatment

$CD34^+CD38^-$ cells from three patients with CML were selected with flow cytometry sorting and were cultured with IM (1 μM), LBH (50 nM), or the combination of IM and LBH or without exposure to drugs (controls) for 24 hr. RNA was extracted, amplified, labeled, and hybridized to Affymetrix HG U133 plus 2.0 Arrays. Microarray data analyses were performed, and differentially expressed genes were identified as described in Experimental Procedures.

(A) The $\log_2$ fold changes in gene expression after IM, LBH, and IM plus LBH treatment compared to controls are shown. The column labeled "interaction" shows the fold elevation or reduction of gene expression with the IM+LBH combination compared to the sum of the effects of IM given alone and LBH given alone. Genes shown are those whose expression is significantly altered as a result of the interaction between IM and LBH ($p < 0.01$; fold change ≥ 3).

(B) GSEA was performed to detect enrichment of predetermined gene sets after IM, LBH, and combination treatment. The normalized enrichment scores (NES) for these gene sets are shown. The column labeled "interaction" shows gene sets that are enriched for genes whose expression is significantly altered as a result of the interaction between IM and LBH. The highest ranked gene sets within the most common functional categories are displayed (FDR < 0.1 with up to 10 gene sets per category).

(C) A summary of regulatory mechanisms that are significantly affected by the interaction of LBH and IM is shown.

Table 1. Results of Gene Set Enrichment Analysis

Gene Sets enriched by Interaction between IM and LBH	NES	FDR q-val
Gene Set Name		
GPCRS_CLASS_A_RHODOPSIN_LIKE	2.334226	<1.00E-05
GPCRDB_CLASS_A_RHODOPSIN_LIKE	2.423674	<1.00E-05
STEMCELL_COMMON_UP	−2.4496958	<1.00E-05
HSC_INTERMEDIATEPROGENITORS_SHARED	−2.3206468	<1.00E-05
RIBOSOMAL_PROTEINS	−2.4317436	<1.00E-05
TRANSLATION_FACTORS	−2.3928194	<1.00E-05
UVC_XPCS_ALL_DN	−2.4149804	<1.00E-05
UVC_HIGH_ALL_DN	−2.4105043	<1.00E-05
REN_E2F1_TARGETS	−2.1447365	<1.00E-05
HDACI_COLON_BUT16HRS_DN	−2.1105642	0.000194
DNA_REPLICATION_REACTOME	−2.0854743	0.000532
HDACI_COLON_BUT12HRS_DN	−2.0248308	0.001158812
CHEN_HOXA5_TARGETS_UP	−1.9763813	0.001988439
DORSAM_HOXA9_UP	−1.9619048	0.001844117
MYC_TARGETS	−1.8888725	0.004696284
SANSOM_APC_LOSS5_UP	−1.8862375	0.005068596
MENSSEN_MYC_UP	−1.8385748	0.008161867
HSA04210_APOPTOSIS	−1.7196336	0.019393332
KENNY_WNT_DN	−1.6921147	0.024098977
FASPATHWAY	−1.6343089	0.035348434
Gene Sets Sharing Transcription Factor Binding Sites, By Transcription Factor		
YY1	−2.1095614	0
E2F	−2.0739126	0.00629868
E2F	−2.0437264	0.00472401
SREBP1	−2.0296636	0.003779208
E2F	−1.9984082	0.004686787
NRF1	−1.9882506	0.004166033
E2F	−1.973179	0.003408572
NRF2	−1.9532624	0.004709931
E2F	−1.944042	0.004037084
ETS1	−1.9379699	0.004403604
E2F	−1.931731	0.004128379
E2F	−1.9269646	0.003885533
E2F	−1.926879	0.00366967
E2F	−1.8943775	0.003984813
E2F	−1.8883003	0.003785573
E2F	−1.8871185	0.003605307
E2F	−1.8828987	0.00344143
E2F	−1.8726895	0.003154644
E2F	−1.8725656	0.00339813
E2F	−1.8696322	0.003146417
PAX3	−1.8358319	0.003565615
GABP	−1.8336416	0.003446761
E2F	−1.826765	0.003335575
MYCMAX	−1.8066974	0.003531206
ELK1	−1.8054509	0.003711315

Gene expression programs in CML $CD34^{+}CD38^{-}$ cells that were selectively altered by interaction between LBH and IM were identified and categorized. Gene sets representing common functional categories were grouped as shown in Figure 7B. The top two gene sets representative of each of these groups (ranked by FDR) are shown in the top half of the table. Gene sets sharing common transcription factor (TF) binding sites that were selectively altered in CML $CD34^{+}CD38^{-}$ cells by interaction between LBH and IM were identified. The top 25 sets based on FDR are shown in the bottom half of the table. NES, normalized enrichment score; FDR, false discovery rate.

Bioconductor (version 2.4). Expression data were normalized with the robust multiarray average (RMA) algorithm, with background adjustment, quantile normalization, and median polish summarization. Probe sets with low expression levels or low variability across samples were filtered. For genes with multiple probe sets, the gene level expression was set to be the median of the probe sets. Linear regression was used to model the gene expression with the consideration of 2 × 2 factorial design and matched samples. Differentially expressed genes were identified by calculating empirical Bayes moderated t-statistic, and p values were adjusted by FDR with the "LIMMA" package. We focused our attention on the "interaction" between IM and LBH to identify genes for which the extra effect of the combination of IM and LBH is significant—that is, the additional effect of the combination drug that cannot be explained by the additive effect of IM and LBH treatment: Interaction = (Combination − LBH) − (IM − Control). Genes significantly altered by interaction between IM and LBH ($p < 0.01$; fold change, 3) were selected. Gene Set Enrichment Analysis (GSEA) was performed with GSEA software version 2.04 (http://www.broadinstitute.org/gsea/) for detection of enrichment of predetermined gene sets with t-scores from all 13,812 genes for 1,263 gene sets in C2 (curated gene sets) category from the Molecular Signature Database (MsigDB) (Subramanian et al., 2005). Gene sets representing common functional categories were categorized and grouped. We also analyzed enrichment of gene sets with common TF binding sites (586 sets) from MsigDB.

Statistics

Data from independent experiments were reported as the mean ± SEM. Student's t test analysis was performed to determine statistical significance.

ACCESSION NUMBER

Microarray data have been deposited in the Gene Expression Omnibus database (accession number GSE20876).

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2010.03.011.

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B.Z., A.S. and R.B. designed research; B.Z., A.S., S.C., and Y.H. performed research; C.H., L.S., D.S., and T.H. contributed reagents/materials; B.Z., A.S., S.C., M.L., K.S., and R.B. analyzed data; and B.Z., A.S., S.C., M.L., K.S., and R.B. wrote the paper.

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