Supplemental Information

Effective Targeting of Quiescent Chronic Myelogenous Leukemia Stem Cells by Histone Deacetylase

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Figure S1, related to Figure 1. Effect of LAQ and IM on histone acetylation in primary CML CD34+ cells. CD34+ cells obtained from the peripheral blood of newly-diagnosed CML patients were cultured for 24 hours in the presence of the indicated concentrations of LAQ and IM. Histone proteins were extracted and analyzed by Western blotting to determine levels of Acetyl-Histone-H3 and total Histone-H3. Representative results are shown in (A). Densitometry analysis was performed and the ratio of Acetyl-Histone-H3 to total Histone-H3 was determined and normalized based on the control. Histone acetylation levels after treatment with LAQ (B) and LAQ with IM (C) are shown and represent the mean ± SEM of 3 CML samples. Significance levels: *p<0.05; **p<0.01 compared with no treatment. (D) CD34+ cells from CML patients were treated with LAQ, IM or LAQ combined with IM for 24 hours, followed by Western blotting to determine BCR-ABL expression.
Figure S2, related to Figure 2. The combination of HDACi and IM induces apoptosis, inhibits proliferation, inhibits CFC, and reduces viability of CML progenitor cells. CD34+ cells from newly diagnosed CML patients (n=5) and healthy donors (n=6; 3 CB and 3 BM samples) were labeled with CFSE and exposed to the indicated concentrations of LAQ and IM for 96 hours. Cells were labeled with Annexin-PE and analyzed by flow cytometry to measure apoptosis (Annexin positive cells) and proliferation (reduction in CFSE fluorescence). Results from a representative experiment are shown in (A). Apoptosis of CML cells treated with LAQ alone is shown in (B), and of CML cells co-treated with LAQ and 1μM IM in (C). Apoptosis of normal CD34+ cells treated with LAQ alone are shown in (D), and normal cells co-treated with LAQ and 1 μM IM in (E). Apoptosis of undivided cells for samples co-treated with LAQ and 1 μM IM is shown for CML samples in (F) and for normal samples in (G). ModFit software was used to determine the proliferation index. All proliferation index values were normalized to the untreated
control. Relative proliferation of CML cells treated with LAQ alone is shown in (H), and proliferation of cells co-treated with LAQ and 1μM IM is shown in (I). The relative proliferation of normal cells treated with LAQ alone is shown in (J), and co-treated with LAQ and 1μM IM in (K). CFC assays were performed on CML and normal CD34+ cells exposed to LAQ at different concentrations, with or without IM for 96 hours. Following exposure cells were plated in methylcellulose progenitor culture and the total number of erythrocytic and granulocytic colonies was enumerated after 14 days. Results shown are normalized to the number of colonies in the untreated control sample. CFC dose response for CML cells treated with LAQ alone is shown in (L), and co-treated with LAQ and 1μM IM in (M). Results for normal cells treated with LAQ alone are shown in (N), and co-treated with LAQ and 1μM IM in (O). Results shown represent the mean ± SEM for multiple samples. Significance levels: *p<0.05, **p<0.01, ***p<0.001 compared with no treatment; † p<0.05; †† p<0.01 compared with IM alone. (P) CML CD34+ cells labeled with CFSE, CD34-APC and CD38-PE, were flow cytometry sorted for CD34+CD38- and CD34+CD38+ cells with uniform CFSE labeling. Cells were cultured for 48 hours with or without IM, LBH, or the combination. The percentage of viable cells was evaluated by Trypan blue labeling. Cell viability was reduced with IM and LBH treatment and was further reduced by treatment with the combination of IM and LBH. Results shown are representative of 2 experiments. (Q) CD34+CD38- cell morphology was evaluated after Wright-Giemsa staining. An increased number of apoptotic bodies and necrotic cells was seen after treatment with the combination of IM and LBH. All scale bars represent a size of 10μm. (R) CD34+CD38- cells were labeled with anti-active Caspase 3 and counterstained with DAPI. The 4 panels show results for untreated controls, IM, LBH and IM+LBH treatment. Each panel shows CFSE, active Caspase 3, and DAPI labeling and the overlay of the 3 labels. An increase in activated Caspase 3 positive cells was seen after treatment with the combination of IM and LBH compared with untreated controls, and cells treated with IM and LBH alone. The arrows (white) identify cells which are both CFSE bright and active Caspase 3 positive after treatment with the IM and LBH combination. All scale bars represent a size of 10μm.
Figure S3, related to Figure 3. The combination of LAQ and IM eliminates CML cells capable of multilineage engraftment in immunodeficient mice. CML CD34+ cells (1x10^6 cells per mouse; 5 mice per condition) and cord blood CD34+ cells (1x10^5 cells per mouse; 5 mice per condition) were cultured for 96 hours in the absence of drug (control), with IM (1μM) alone or IM in combination with LAQ (50nM) and then transplanted into NSG mice. Mice were euthanized after 6 weeks and BM cells were analyzed by flow cytometry. Representative results for CML cells are shown in (A). Combined results for engraftment of CML cells in BM are shown in (B). BCR-ABL mRNA levels in mouse BM analyzed using Q-PCR analysis are shown in (C). Representative results for cord blood cells are shown in (D). Combined results for engraftment of cord blood cells in BM are shown in (E). Results represent the mean ± SEM for multiple samples. Significance levels: *p<0.05, **p<0.01, ***p<0.001, compared with no treatment.
Figure S4, related to Figure 5. The combination of LBH and IM results in modest systemic toxicity and reduction in blood cell and progenitor counts in wild-type mice. Wild-type FVB/N mice (6 mice per condition) were treated with IM (200mg/kg daily by gavage), LBH (30 mg/kg body weight intraperitoneally 3 days per week on Monday, Wednesday and Friday), the combination of LBH with IM, or vehicle alone (controls) for 4 weeks. Mice were followed for clinical signs of toxicity. Body weight was measured every week. Results for body weight measurements (normalized to body weight at start of treatment) are shown in panel (A). Mice were euthanized after 4 weeks of treatment and PB and BM cells were obtained. Results for peripheral blood WBC (B), RBC (C), and platelets (D), and BM GMP (E), MEP (F), and Lin-Scal-1+Kit+ (LSK) stem cells (G) are shown. Significance levels: IM+LBH compared to control p<0.001 for WBC, p<0.005 for RBC, p<0.05 for platelets, p<0.01 for GMP, p<0.05 for MEP, p=NS for LSK.
Supplemental Experimental Procedures

Analysis of cell viability. CML CD34+ cells were labeled with 1.25µM CFSE for 10 minutes at 37°C, incubated overnight in Stemspan serum-free medium supplemented with low concentrations of growth factors to release unbound CFSE, then labeled with CD34-APC and CD38-PE, and flow cytometry sorted for CD34+CD38- and CD34+CD38+ cells with uniform CFSE labeling. Cells were then cultured for 48 hours with or without IM, LBH, or the combination. Cells were labeled with Trypan Blue and percentage of viable cells (Trypan blue negative) was evaluated. Cells were also spread on slides and labeled with Wright-Giemsa stain and evaluated for morphology, specifically for the presence of apoptotic bodies and necrotic cells. To measure Caspase 3 activity, cells were fixed, permeabilized and labeled with anti-active Caspase 3, counterstained with DAPI, and analyzed by fluorescent microscopy.

Analysis of apoptosis and cell cycle in stem cells in vivo. To evaluate the effect of drug administration on apoptosis of stem cells in vivo, a set of leukemic mice were sacrificed on day 5 of treatment and apoptosis in the LSK population was assessed by labeling with Annexin V and DAPI. To evaluate stem cell cycling, mice were injected intraperitoneally with 5-ethynyl-2'-deoxyuridine (EdU; 1 mg of EDU/animal; Invitrogen) and sacrificed 2 hours later. BM cells were labeled with surface markers for stem cells, fixed, permeabilized, and labeled with Alexa Fluor 647 azide and 4',6-diamidino-2-phenylindole (DAPI) using the Click-iT™ EdU Alexa Fluor® 647 Flow Cytometry Assay kit (Invitrogen, Carlsbad, CA). EdU incorporation in LSK cells was measured by flow cytometry.