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Sulforaphane Potentiates the Efficacy of Imatinib against Chronic Leukemia Cancer Stem Cells through Enhanced Abrogation of Wnt/β-Catenin Function

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Supporting Information

ABSTRACT: Sulforaphane (SFN) has been indicated for the prevention and suppression of tumorigenesis in solid tumors. Herein, we evaluated SFN's effects on imatinib (IM)-resistant leukemia stem cells (LSCs). $CD34^+/CD38^-$ and $CD34^+/CD38^+$ LSCs were isolated from KU812 cell line flowcytometrically. Isolated LSCs showed high expression of *Oct4*, *CD133*, *β-catenin*, and *Sox2* and IM resistance. Differentially, $CD34^+/CD38^-$ LSCs demonstrated higher BCR-ABL and β-catenin expression and imatinib (IM) resistance than $CD34^+/CD38^+$ counterparts. IM and SFN combined treatment sensitized $CD34^+/CD38^-$ LSCs and induced apoptosis, shown by increased caspase 3, PARP, and Bax while decreased Bcl-2 expression. Additionally, the combined treatment reduced BCR-ABL and β-catenin and MDR-1 protein expression. Mechanistically, IM and SFN combined treatment resensitized LSCs by inducing intracellular reactive oxygen species (ROS). Importantly, β-catenin-silenced LSCs exhibited reduced glutathione *S*-transferase pi 1 (GSTP1) expression and intracellular GSH level, which led to increased sensitivity toward IM and SFN. We demonstrated that IM and SFN combined treatment effectively eliminated $CD34^+/CD38^-$ LSCs. Since SFN has been shown well tolerated in both animals and human, this regimen could be considered for clinical trials.

KEYWORDS: *imatinib resistance, leukemia stem cells, sulforaphane, β-catenin, reactive oxygen species*

INTRODUCTION

Chronic myeloid leukemia (CML) is a hematopoietic malignancy characterized by the BCR-ABL-fusion gene, a product of a reciprocal translocation of chromosome 9 and 22, or the so-called Philadelphia chromosome, Ph. CML can be categorized into three phases. The chronic phase (CP) is characterized by the sole genetic abnormality Ph^+ in myeloid cells. Subsequently, CP transforms into the accelerated phase (AP), which is manifested by an increased number of blast cells in the bone marrow and peripheral blood. Shortly, AP progresses into the blast crisis (BC), where numerous additional genetic aberrations are detected and there is increasing resistance to therapy.

The current first line therapy for CML is BCR-ABL kinase inhibitors, imatinib mesylate (or Gleevec), which has been shown highly effective in most patients in the chronic phase of the disease.[1] However, an estimated 7% of CML patients who receive imatinib demonstrate progression to accelerated-phase CML or blast crisis after 5 years of treatment, and patients with advanced disease do not respond well to imatinib and exhibit frequent relapse.[2] It is known that clinical imatinib resistance and relapse in CML patients have been attributed to various mutations within the coding region of BCR-ABL-kinase, typified by T315I mutant.[3] Though some of these mutations were thought to develop under the selection pressure of imatinib therapy, some imatinib-naive patients harbor mutant BCR-ABL transcripts.[4,5] In addition, low levels of imatinib-resistant cells have been shown to exist even prior to imatinib treatment, and these cells may represent clonal diversity of Ph^+

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hematopoiesis at the time of disease diagnosis.[6] Upon the clearance of the major Ph^+ clone during imatinib therapy, the drug-resistant minor clones may have a growth advantage and replace normal hematopoiesis. Recently, leukemia stem cells (LSCs) have been identified and isolated from CML patients using cell surface markers such as CD34 and CD38.[7] It has been suggested that two major subpopulations of LSCs exist in CML patients, namely, the $CD34^+/CD38^-$ and $CD34^+/CD38^+$.[8] Additional evidence indicates that $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells represent the LSCs in chronic-phase and blast-crisis CML respectively and BCR-ABL was constitutively activated in both cell populations. While activated BCR-ABL signaling may support the initiation and proliferation of CML, it has been shown that LSCs may be inherently insensitive to BCR-ABL kinase inhibitors due to BCR-ABL-independent mechanisms.[9] Taken together, an emerging hypothesis states that LSCs may not be BCR-ABL addicted and the development of novel therapeutic strategies either alone or in combination with kinase inhibitors might be more effective in targeting LSCs.

Dietary consumption of cruciferous vegetables such as broccoli and cabbage is thought to provide a chemo-preventative benefit against several types of cancer due to the high level of isothiocyanates.[10] Sulforaphane (SFN, 4-methylsufinylbutyl isothiocyanate) represents one of the most abundant naturally occurring members of the isothiocyanate family.[11] At low concentration, SFN is a well-characterized inducer of several phase II detoxification enzymes including glutathione *S*-transferases and quinone reductase.[12] However, at high concentration, SFN has been reported to cause growth inhibition and induction of apoptosis in a variety of human cancer types.[13] One of the mechanisms by which SFN induces its anticancer effects is the induction of reactive oxygen species (ROS) and depletion of intracellular glutathione pool leading to the lowering of the oxidative stress threshold and apoptosis.[14] A small scale clinical trial has demonstrated that no significant toxicity was found associated with high dosage of glucosinolate, or isothiocyanate, suggesting the safety of SFN as a potential candidate for cancer treatment.[15] However, the effects of SFN on self-renewal and therapy resistance of leukemia stem cells have not yet been explored.

In this study, we employed the flow cytometric technique to isolate $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells from a human myelogenous leukemia cell line, KU812. We characterized these two CML subpopulation cells and revealed their high expression of stemness genes and marked imatinib resistance, demonstrating LSC properties. Subsequently, we investigated sulforaphane as an anti-LSC agent and provided experimental evidence that SFN sensitized imatinib-resistant LSCs, depleted intracellular GSH concentration, and induced apoptosis. Thus, SFN could be considered as a potential adjuvant therapeutic agent in treating imatinib-resistant CML patients in the future.

MATERIALS AND METHODS

Reagents. D,L-Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane, >99% pure) was obtained from LKT Labratories (St Paul, MN); *N*-acetyl-L-cysteine (NAC) and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (St. Louis, MO). TRIzol reagent, fetal bovine serum (FBS), and trypsin-EDTA (T/E) were obtained from Gibco BRL Life Technologies (Grand Island, NY). QIAGEN One-tube RT-PCR system and Annexin-V/FITC apoptosis kits were obtained from Qiagen Inc. (Valencia, CA) and R&D Systems, Inc. (Minneapolis, MN) respectively. Lipofectamine was purchased from Invitrogen (Rockville, MD). An enhanced chemiluminescence (ECL) kit was purchased from Amersham Corp. (Arlington Heights, IL). Anti-pCrkL and anti-Bcr-abl antibodies were purchased from Chemicon International, Inc. (Temecula, CA). Anti-Bcl2, anti-Bax, antiphosphorylated STAT5 (anti-pSTAT5), anti-GSTP1, and anti-β-actin antibodies were purchased from Novus Biologicals (Littleton, CO).

Cell Culture and Gene Silencing. Human leukemia KU812 cell line was obtained from American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, in an incubator with humidified air at 37 °C with 5% CO_2. As an untreated solvent control, cells were treated with DMSO at a final concentration of <0.05%. KU812 cells were transfected with control siRNA and siRNA for β-catenin (sc-29209, Santa Cruz Biotechnology, USA). Transfection was performed according to the vendor's instructions. Cells were harvested 48 h post transfection for experiments.

Flow Cytometric Analysis and Isolation of Leukemia Stem Cells. KU812 cells were enriched for $CD34^+$ cells using magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA) and labeled with CD34-PE-CY5, CD38-FITC, or isotype control antibodies (all from Coulter- Immunotech Co., Miami, FL, USA). Cells were analyzed and sorted on a FACSAria Cell Sorter unit (Becton Dickinson), using propidium iodide (PI) as a viable stain. Cells were gated on low side scatter, low-to-moderate forward scatter, and low PI. For data acquisition, at least 10,000 events were analyzed. The purity and viability of isolated cells were routinely >98%.

Trypan Blue Exclusion Assay. Cellular viability of KU812 bulk parental cells, $CD34^+/CD38^+$ and $CD34^+/CD38^-$ subpopulations was evaluated using the trypan blue exclusion method. Briefly, cells were treated with different concentrations of imatinib (from 0 to 1.0 μM) and sulforaphane (0 to 30 μM) for 24 h. Cells were then harvested, followed by trypan blue staining, and viable cells were counted using light microscopy. For each imatinib and/or sulforaphane concentration, the cell count was performed in triplicate and the average value was obtained. Viability was calculated as follows: cell viability (%) = (viable cell number$_{test}$/viable cell number$_{control}$) × 100%.

Flow Cytometric Assessment of Apoptosis Using Annexin V/PI Assay. Apoptosis was assessed by evaluation of Annexin V binding to phosphatidylserine (PS) that was externalized in the early stage of apoptosis. Annexin V binding to cells was determined using a commercially available Annexin V apoptosis detection kit and flow cytometry. The percentage distributions of apoptotic cells were calculated by CellQuest software (Becton, Dickinson and Co., San Jose, CA).

RNA extraction and RT-PCR. The mRNA levels of *CD133*, *Oct4*, *β-catenin*, and *SOX2* were quantified by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), using *GAPDH* mRNA as an internal control. Briefly, KU812 parental and different subpopulation cells were cultured in 100 mm tissue culture dishes and total RNA was extracted with a TRIzol RNA isolation kit. RNA concentration and purity were determined based on measurement of the absorbance at 260 and 280 nm. Please refer to the Supporting Information for all the primer sequences. From each sample, 250 ng of RNA was reverse-transcribed, using 200 U of SuperScript II RNase-H reverse transcriptase, 20 U of RNase inhibitor, 0.6 mM of dNTP, and 0.5 mg/mL of oligo (dT). PCR analyses then were performed on the aliquots of the cDNA preparations to detect *CD133*, *Oct4*, *SOX2*, *β-catenin*, and *GAPDH* (as an internal standard) gene expression, using the FailSafe PCR system (Epicenter Technologies, Madison, WI). The reactions were carried out in a volume of 50 μL, containing a final concentration of 50 mmol/L Tris−HCl, (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MnCl2, 0.2 mmol/L dNTP, 2 U of Taq DNA polymerase, and 50 pmol of primers. After initial denaturation for 2 min at 95 °C, 26 cycles of amplification (at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min) were performed, followed by a 7 min extension at 72 °C.

Western Blotting. Total protein lysates were obtained from KU812 parental cells and different subpopulations (untreated and/or treated with different concentrations of imatinib and sulforaphane) using routine extraction protocol. Briefly, cells were harvested and

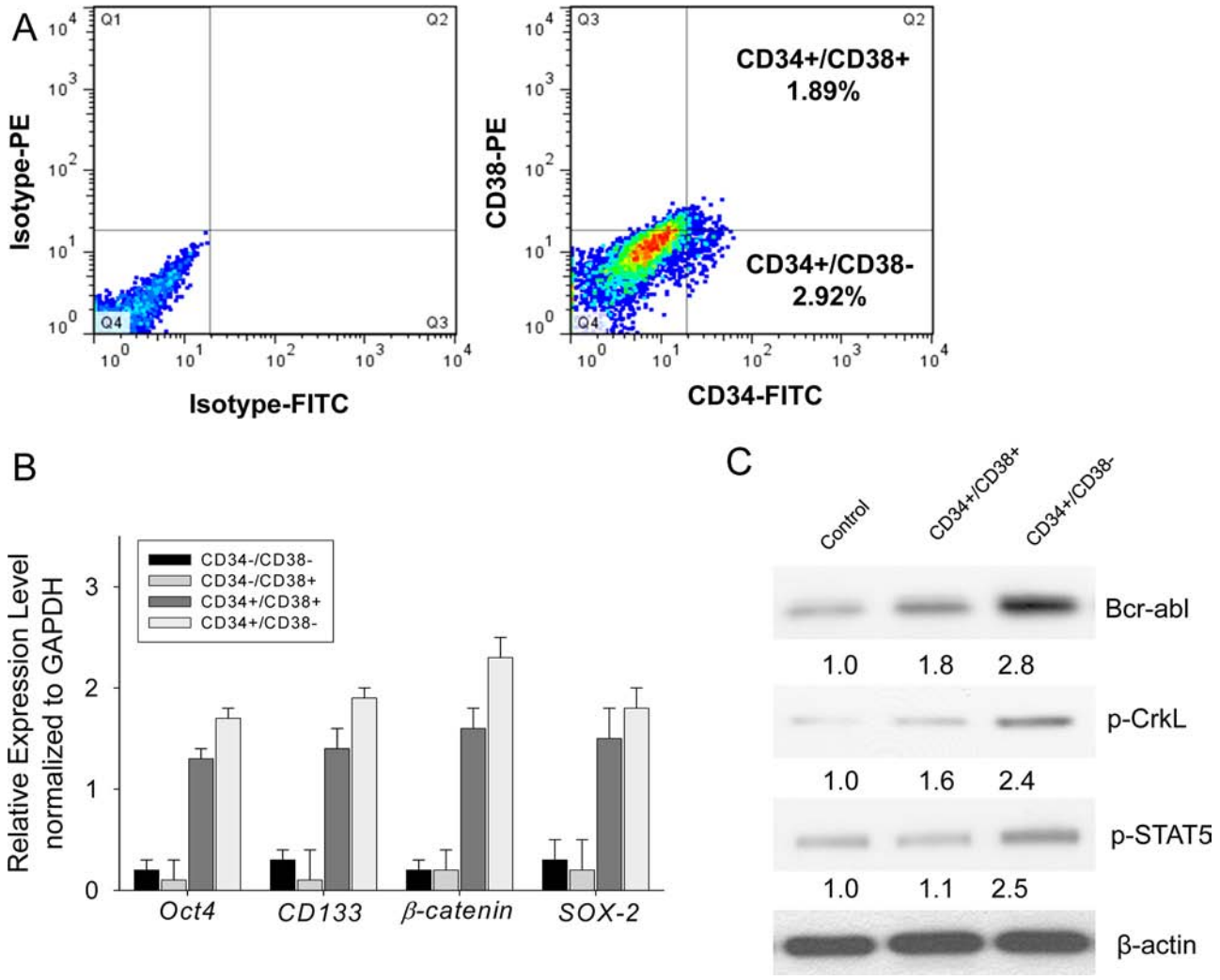

Figure 1. Identification and characterization of leukemia stem cell (LSC) populations in the KU812 cell line. (A) Two populations of LSCs were identified, $CD34^+/CD38^+$ (1.89%) and $CD34^+/CD38^-$ (2.92%), using the FACS technique. (B) Stemness gene expression in LSCs. Messenger RNA level of 4 stemness genes including *Oct4*, *CD133*, *β-catenin*, and *SOX-2* were found elevated in the two isolated LSCs ($CD34^+$) while the parental KU812 cells ($CD34^-$) demonstrated a low level of stemness gene transcripts. $CD34^+/CD38^-$ cells appeared to contain a significantly higher level of *β-catenin* mRNA as compared to that of $CD34^+/CD38^+$ cells. (C) Comparative CML signaling profile between 2 populations of LSCs. $CD34^+/CD38^-$ cells were found to express a higher level of BCR-ABL, phospho-CrkL and phospho-STAT5 than that of $CD34^+/CD38^+$ counterparts. Numbers indicate signal intensity, expressed in fold change relative to the control.

lysed in lysis buffer followed by centrifugation at 10000*g* for 30 min at 4 °C. Protein concentration was quantified using Bio-Rad detergent-compatible protein assay reagent (Bio-Rad Laboratories; Hercules, CA), and 50 μg of proteins was separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes using standard protocol. The membranes were blocked in 5% powdered skimmed milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and incubated with primary antibodies overnight at 4 °C. Membranes were washed with TBST 3 times and incubated with horseradish peroxidase-labeled secondary antibody for 45 min at room temperature followed by 3 washes with TBST. Signal detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents.

Detection of Intracellular ROS and GSH Contents. Intracellular ROS contents were measured by flow cytometry following staining with DCF. Drug treated and untreated cells were incubated with DCF (10 μM) for 20 min, washed with PBS, and analyzed using a flow cytometer. Intracellular GSH content was measured using a commercial glutathione assay kit (Calbiochem, San Diego, CA) according to the protocol provided by the vendor. The cellular GSH content was converted to total cellular protein, and KU812 and KU812 LSCs were pelleted and lysed as described as above. The protein concentration was then determined using the Bradford standard method (Bio-Rad Laboratories; Hercules, CA).

Statistical Analysis. Each experiment was performed in triplicate. The results were expressed as means ± SD. The significant difference between imatinib and/or SFN-treated and vehicle-treated control groups was analyzed by *t* test. Asterisks indicate that the values are significantly different from the control (*, $p < 0.05$; **, $p < 0.01$).

RESULTS AND DISCUSSION

Isolation and Characterization of Leukemia Stem Cells (LSCs). The inhibition of BCR-ABL signaling using kinase inhibitors for the treatment of Philadelphia-positive (Ph^+) chronic myeloid leukemia (CML) represents a highly effective method for controlling the disease but not a cure. This is mainly due to the failure of these kinase inhibitors to target and eliminate leukemia stem cells (LSCs). LSCs have been shown to be responsible for disease progression and/or relapse in patients who receive imatinib treatment. Therefore, the discovery of new or alternative agents which can be used to target these LSCs and/or increase the sensitivity of currently

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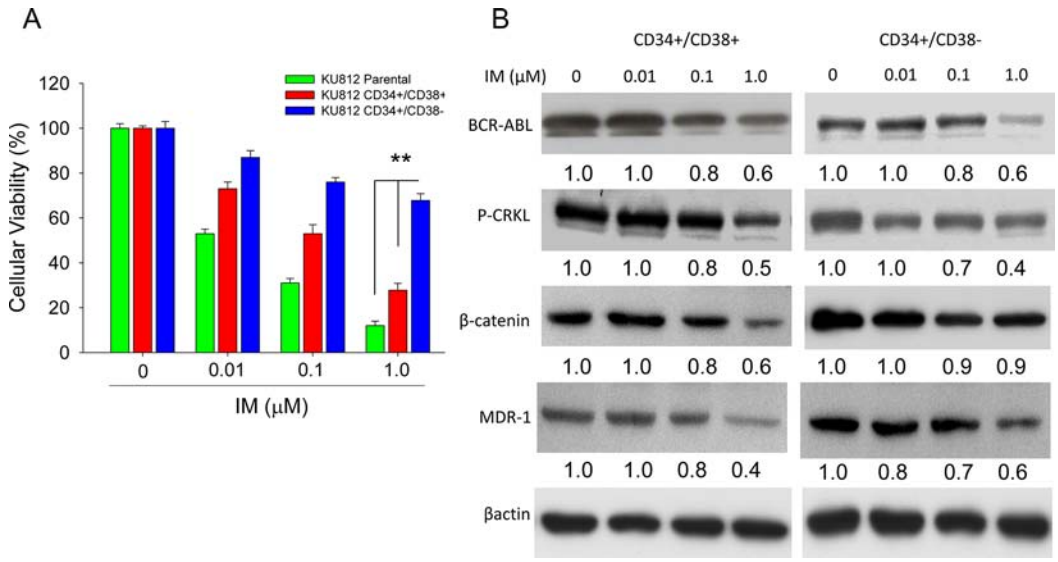

Figure 2. Comparative imatinib resistance and expression profiles between $CD34^+/CD38^-$ and $CD34^+/CD38^+$ LSCs. (A) Two subpopulations of LSCs were treated with various concentrations of imatinib (IM) for 24 h and examined for their IM resistance. $CD34^+CD38^-$ LSCs were significantly more resistant to IM treatment. Even at 1 μM IM, 76 ± 4% of $CD34^+CD38^-$ cells remained viable as compared to 27 ± 2% in $CD34^+CD38^+$ counterparts and 12 ± 2% in parental cells. (B) BCR-ABL signaling in $CD34^+CD38^-$ and $CD34^+CD38^+$ LSCs was suppressed by IM treatment in a dose-dependent manner as shown by Western blots. β-Catenin and MDR-1 expression was not significantly affected by IM treatment in the $CD34^+CD38^-$ LSCs.

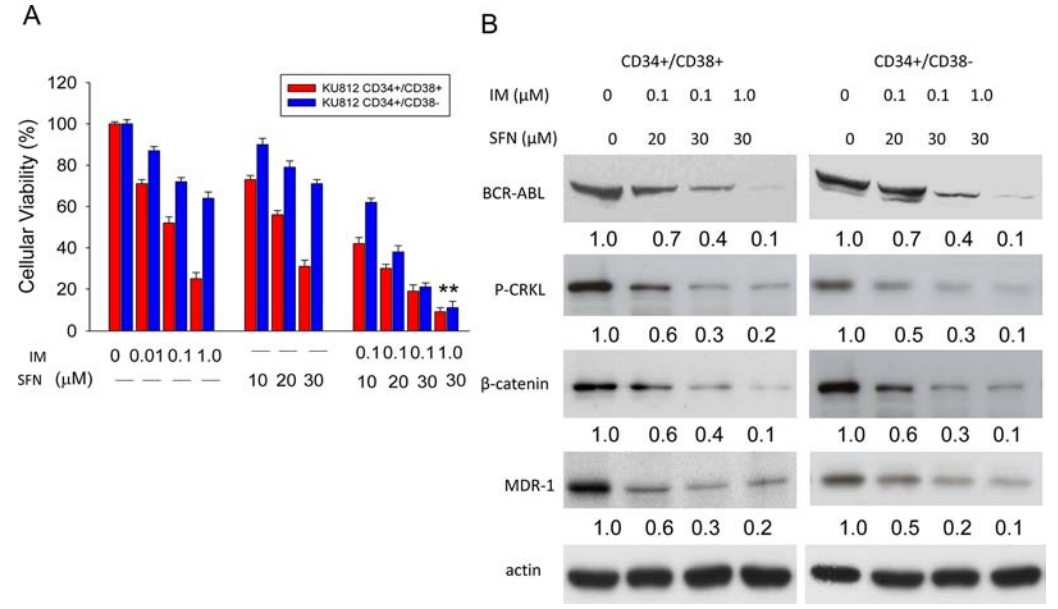

Figure 3. Sulforaphane (SFN) increased imatinib (IM) sensitivity in $CD34^+/CD38^-$ LSCs. (A) $CD34^+/CD38^-$ LSCs were significantly more resistant to imatinib (1 μM) as compared to their $CD38^+$ counterparts (76 ± 4% versus 27 ± 2%). Similarly, $CD34^+/CD38^-$ LSCs were more resistant to sulforaphane (30 μM) than their $CD38^+$ counterparts (70 ± 2% versus 36 ± 2%). However, the combination of IM and SFN overcame the drug resistance and eliminated both LSCs. **, $p < 0.05$ compared to single IM and SFN treatment group. (B) SFN and IM combination treatment not only significantly downregulated BCR-ABL signaling but also decreased β-catenin and MDR-1 expression in imatinib-resistant $CD34^+CD38^-$ and $CD34^+CD38^+$ LSCs. Numbers indicate signal intensity ratio compared to untreated control.

available tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, dasatinib, and bosutinib, may represent the ultimate cure for CML. Here, we used established LSC markers CD34 and CD38 to isolate LSCs from a human myelogenous leukemia cell line, KU812. Our data demonstrated that the proportions of $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells within KU812 cells were 1.89% and 2.92%, respectively (Figure 1A). Subsequently, these two population cells were characterized based on the expression of stemness genes. The expression level of stemness genes *Oct4*, *CD133*, *β-catenin*, and *Sox-2* were found significantly higher in the $CD34^+/CD38^-$ cell population as compared to those in the $CD34^+/CD38^+$ and $CD34^-$ populations (Figure 1B). This finding indicated that different subpopulations of $CD34^+$ cells existed and their genetic difference might contribute to different degrees of drug resistance. This is in agreement with previous reports that β-catenin was found overexpressed in the granulocyte macrophage progenitors from blast-crisis CML patients and mice.[16,17]

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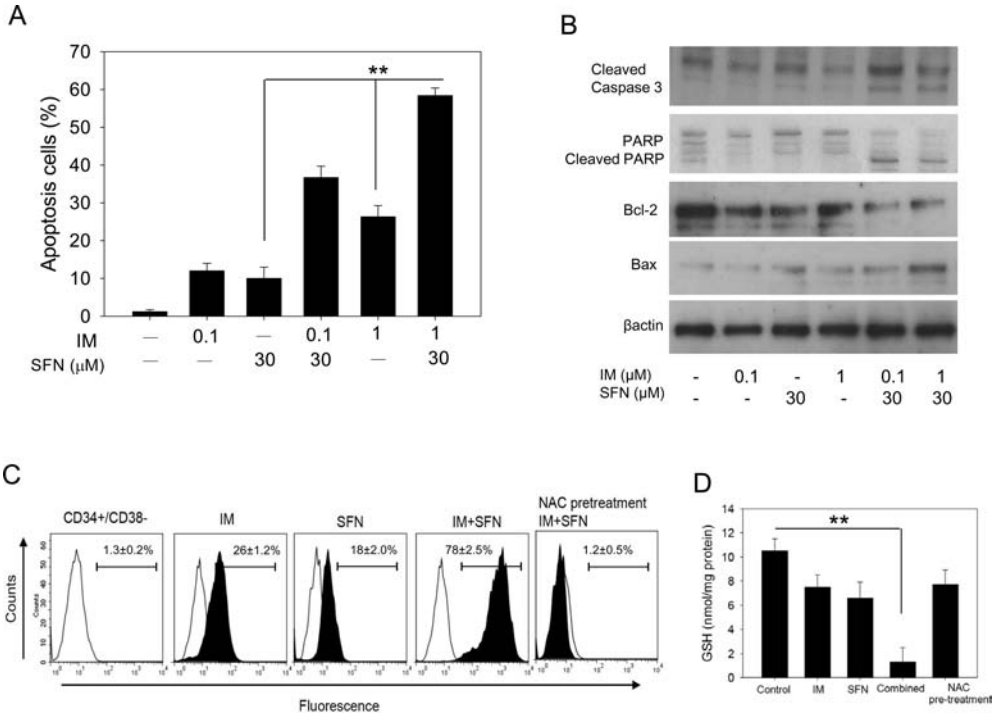

Figure 4. Combined imatinib (IM) and sulforaphane (SFN) induced ROS-mediated apoptosis in $CD34^+/CD38^-$ LSCs. (A) IM and SFN single treatment led to a relatively low extent of apoptosis in $CD34^+/CD38^-$ LSCs. However, when combined, approximately 55% apoptosis could be achieved. (B) IM and SFN combined treatment induced apoptosis in $CD34^+/CD38^-$ LSCs. The combined treatment resulted in the increased level of cleaved caspase 3 and PARP, as well as an increased level of proapoptotic molecule, Bax. In contrast, pro-survival Bcl-2 expression was suppressed under the combined treatment. (C) IM and SFN combined treatment induced apoptosis via increasing intracellular ROS in LSCs as demonstrated by the increase in DCF fluorescence. IM+SFN-induced ROS were suppressed by the pretreatment of an antioxidant NAC. GSH levels in $CD34^+/CD38^-$ cells treated with 30 μM SFN. (D) The intracellular GSH contents were quantitatively measured under different treatments. The combined treatment significantly reduced the total GSH contents by approximately 7-fold as compared to either IM or SFN single treatment.

Enhanced BCR-ABL signaling is the hallmark of LSCs; thus we examined BCR-ABL expression in both $CD34^+$ subpopulations of KU812 cells. It was observed that the expression levels of BCR-ABL and its substrate, phosphorylated CrkL (p-CrkL), were relatively the highest in $CD34^+/CD38^-$ cells followed by $CD34^+/CD38^+$ and parental cells (Figure 1C). Furthermore, overtly activated β-catenin signaling has been shown to contribute to drug resistance in leukemic stem cells; notably, MLL LSCs that have acquired resistance against GSK3 inhibitors could be resensitized by suppression of β-catenin expression.[18,19] The differential expression level of BCR-ABL/p-CrkL/β-catenin was used as an additional distinguishing signature for two $CD34^+$ cell populations of LSCs in our study.

$CD34^+/CD38^-$ LSCs Are Resistant to Imatinib Treatment. Two LSC populations isolated were subjected to imatinib treatment. As expected, both were found significantly more resistant to imatinib treatment when compared to the parental KU812 cells (Figure 2A). It was apparent that $CD34^+/CD38^-$ cells were relatively insensitive to imatinib treatment even at the concentration of 1 μM (approximate 76 ± 4% viability) while $CD34^+/CD38^+$ demonstrated less resistance toward 1 μM imatinib treatment (approximate 27 ± 2% viability). Next, we examined the possible molecular pathways involved in imatinib resistance. First, the expression level of BCR-ABL and phospho-CrkL was compared in imatinib-treated $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells. Imatinib treatment resulted in a dose-dependent suppression of BCR-ABL and p-CrkL in both cell populations (Figure 2B). It appeared that $CD34^+/CD38^-$ endogenously expressed a higher level of both β-catenin and MDR-1 when compared to their $CD34^+/CD38^+$ counterparts (Figure 2B). Interestingly, imatinib treatment did not appear to significantly suppress the expression level of β-catenin and MDR-1 in particular in $CD34^+/CD38^-$ cells as compared to that in $CD34^+/CD38^+$ counterparts (Figure 2B). This result suggested that the differential expression of intrinsic β-catenin and MDR-1 could be the molecular determinants for imatinib resistance between the two LSC populations.

Sulforaphane Treatment Increased Imatinib Sensitivity in $CD34^+$ LSCs. Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables, has received much attention because it has been shown to be an effective chemoprotective and preventive agent in cell culture, carcinogen-induced and genetic animal cancer models.[20] Based on these properties, we aimed to evaluate if SFN could target and eliminate LSCs. First, we demonstrated that $CD34^+/CD38^-$ cells were highly resistant to imatinib treatment such that approximately 76 ± 4% of cells remained alive even at high concentration (1 μM) as compared to 27 ± 2% in $CD34^+/CD38^+$ counterparts (Figure 3A). Similarly, an estimated 36 ± 2% and 70 ± 2% of viable cells were detected in $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells respectively, under high concentration of SFN (30 μM, Figure 3A). Interestingly, the addition of SFN significantly increased imatinib sensitivity in $CD34^+/CD38^-$ (β-catenin high) cells (Figure 3A). For instance, the addition of 30 μM SFN in the presence of 1 μM imatinib led to the elimination of approximately 88% (12 ± 2% viability) of $CD34^+/CD38^-$ cells as compared to 24% (76 ± 4% viability) without SFN. We also examined the effects of IM and SFN combined treatments on BCR-ABL signaling. The combined treatment

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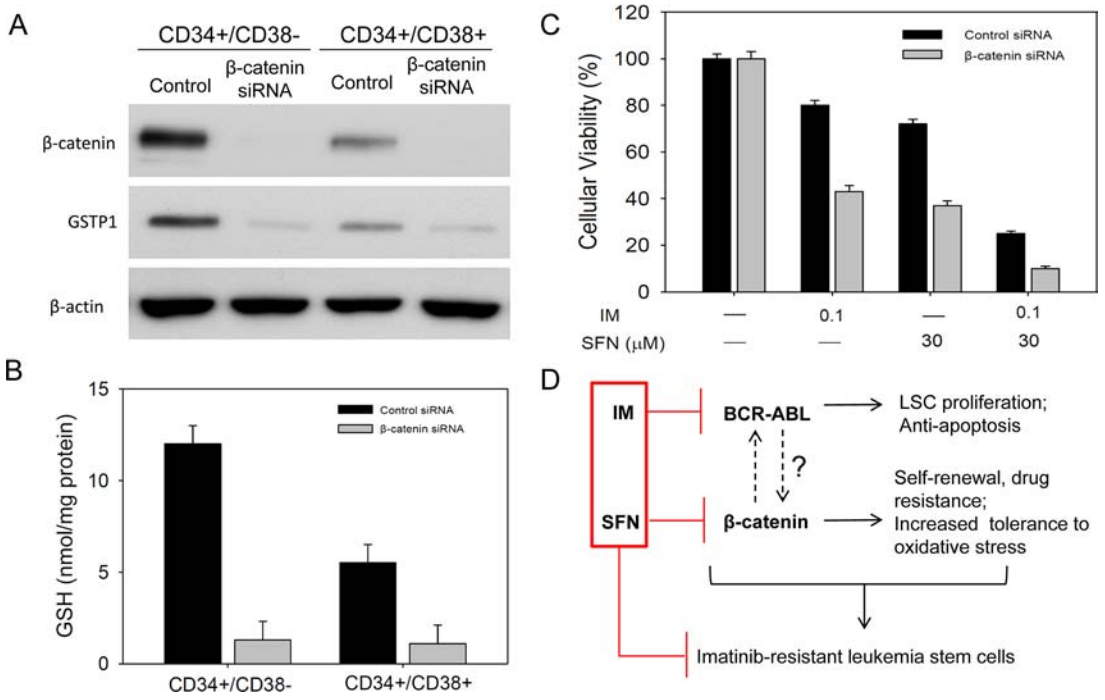

Figure 5. Downregulation of β-catenin compromised ROS modulating ability of LSCs and increased drug sensitivity. (B) β-Catenin was silenced in both $CD34^+$ LSC populations using the siRNA technique. β-Catenin downregulation was accompanied by the decreased expression of GSTP1. This was reflected by the decreased intracellular GSH level in both LCSs. (C) β-Catenin knockdown led to the increased sensitivity of both IM and SFN in $CD34^+/CD38^-$ LCS. (D) Proposed mechanisms involved in IM and SFN combined treatment.

could significantly reduce the expression of BCR-ABL in a dose-dependent manner (Figure 3B). In addition, the level of phosphorylated CrkL, the substrate of BCR-ABL kinase, was also severely suppressed by the combined treatment by 80% and 90% in $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells respectively (Figure 3B). More importantly, β-catenin expression was markedly inhibited in both LSCs as well as MDR-1 expression. Differentially, SFN treatment did not significantly suppress BCR-ABL signaling to the comparable level as imatinib did whereas SFN treatment downregulated the expression of β-catenin and MDR-1, implicating its role in targeting LSCs and decreasing drug resistance. These findings indicated that the combined treatment of IM and SFN effectively downregulates self-renewal and drug resistance in LSCs.

Sulforaphane Induced Apoptosis in Imatinib-Resistant $CD34^+/CD38^-$ LSCs. SFN has been reported to prevent tumor development and suppress cancer progression via a broad spectrum of molecular mechanisms. These include modulation of phase I and phase II detoxification pathways as well as induction of apoptosis, cell cycle arrest.[21] However, studies on the potential therapeutic effects of SFN in targeting drug-resistant LSCs have been limited. Here we provided evidence that SFN exerted its anti-LSC effects via the induction of oxidative stress and apoptosis. According to our data, SFN alone was not as effective as imatinib on eliminating either bulk CML cells or $CD34^+$ LSCs. However, the addition of SFN appeared to sensitize LSCs toward imatinib. Importantly, we have isolated two LSC populations, namely, $CD34^+/CD38^-$ and $CD34^+/CD38^+$, which represent the primitive stem cells in chronic-phase CML and the more differentiated progenitor cells in blast-crisis CML, respectively.[22,23] Having established that SFN treatment significantly increased imatinib sensitivity in $CD34^+/CD38^-$ (β-catenin high) LSCs, we subsequently examined if this increased sensitivity was correlated to increased apoptosis. We found that single treatment of either IM (1 μM) or SFN (30 μM) contributed to approximately 26 $\pm$ 2.0% and 10 $\pm$ 3.0% apoptosis in $CD34^+/CD38^-$ LSCs respectively (Figure 4A). However, when combined (1 μM IM and 30 μM SFN), the rate of apoptosis was increased to approximately 58 $\pm$ 2.0% (Figure 4A). The increased incidence of apoptosis was dose-dependent and accompanied by the increased expression of caspase 3, cleaved PARP, and Bax and decreased expression of Bcl-2 (Figure 4B). This finding was in agreement with previous reports that SFN triggered apoptosis in different solid tumors.[24,25]

Sulforaphane and Imatinib Combination Treatment Sensitized $CD34^+/CD38^-$ LSCs via the Induction of Oxidative Stress and the Depletion of Intracellular GSH Contents. It has been shown that BCR-ABL signaling leads to proliferation advantage in CML cells but it also induces rapid accumulation of reactive oxygen species (ROS) via stimulation of the mitochondrial respiratory chain, and this redox imbalance represents a potentially exploitable target in CML cells.[26] SFN has been known to induce apoptosis in different cancer cells via a variety of different mechanisms including eliciting intracellular oxidative stress.[13] Here, we demonstrated that single treatment of either IM or SFN triggered a moderate level of intracellular ROS accumulation, 26 $\pm$ 1.2% and 18 $\pm$ 2.0% respectively (Figure 4C). However, a marked increase of intracellular ROS (78 $\pm$ 2.5%) was observed in IM-resistant $CD34^+/CD38^-$ LSCs receiving combined IM and SFN treatment (Figure 4C). This observation was also reflected by the decrease of intracellular GSH contents (decreased from 10.4 $\pm$ 0.5 nmol/mg to 1.6 $\pm$ 0.6 nmol/mg GSH, Figure 4D). This increase of intracellular ROS could be prevented by the pretreatment of antioxidant, NAC. Our finding was in agreement with a previous report where

sulforaphane generates reactive oxygen species leading to mitochondrial perturbation and subsequent apoptosis in U-937 leukemia cells.[27] In addition, sulforaphane-mediated antiproliferative and apoptosis-inducing abilities were reported to occur at high concentrations ranging from 10 to 30 μM.[28] Here, we demonstrated that single treatment of imatinib or SFN led to a slight increase in the intracellular ROS level in $CD34^+/CD38^-/\beta\text{-catenin}^{high}$ cells; only when imatinib was combined with SFN, the intracellular ROS level was markedly elevated in $CD34^+/CD38^-/\beta\text{-catenin}^{high}$ LSCs. Interestingly, it has been shown that overt β-catenin signaling contributes to the survival and drug resistance in LSCs and hematopoietic stem cells, under the response to physiologic oxidative stress through ROS scavenge.[29,30] This intrinsic source of ROS has been attributed to the activity of BCR-ABL oncogene product and the transformation of normal hematopoietic stem cells.[31] Our data is consistent with these reports that we observed growth inhibition and induction of apoptosis with high concentrations of SFN (30 μM) in $CD34^+/CD38^-/\beta\text{-catenin}^{high}$ LSCs.

β-Catenin Contributed to Drug Resistance in LSCs Was Partially Attributed to the Regulation of GSTP1 Expression Level. It has been demonstrated that β-catenin upregulation is accompanied by the increase in glutathione *S*-transferase pi (GSTP1) in different cancer types and the overexpression of β-catenin and GSTP1 is correlated to IM resistance in CML patients.[32,33] We decided to determine if IM resistance could be reversed by downregulating β-catenin expression in LSCs. When β-catenin gene expression was knocked down using the siRNA technique, the GSPT1 expression level was decreased accordingly (Figure 5A). Consistently, β-catenin-silenced LSCs (in both $CD34^+/CD38^-$ and $CD34^+/CD38^+$ populations) exhibited a decrease in GSTP1 expression which was accompanied by a significantly decreased intracellular GSH level. In addition, the intracellular GSH contents were also drastically reduced in β-catenin-silenced $CD34^+/CD38^-$ and $CD34^+/CD38^+$ LSCs (approximately 6-fold decrease, Figure 5B). Furthermore, β-catenin-silenced $CD34^+/CD38^-$ LSCs became sensitive toward IM and SFN treatments. For instance, $CD34^+/CD38^-$ (high β-catenin) LSCs was relatively insensitive to 0.1 μM IM (80 $\pm$ 2% survival) whereas their β-catenin-downregulated counterparts exhibited an increased sensitivity (42 $\pm$ 2% survival) toward IM (Figure 5C); a similar observation was obtained in SFN treatment where β-catenin-silenced $CD34^+/CD38^-$ LSCs demonstrated an approximately 50% increase in SFN sensitivity. With the combined IM and SFN treatment, 90% of $CD34^+/CD38^-$ LSCs were eliminated (viability 10 $\pm$ 1%). Based on our data, we proposed that the combination treatment of IM and SFN exerted its anti-LSC actions via the following potential mechanisms. IM suppressed the proliferation of LSCs mainly by suppressing BCR-ABL (also β-catenin expression but to a lesser extent). The presence of SFN significantly suppressed β-catenin expression as well as GSTP1 thereby suppressing self-renewal ability and lowering ROS tolerance toward both agents. The ROS level generated by both agents subsequently induces apoptosis in LSCs (Figure 5D).

Isothiocyanates are found at high levels in cruciferous vegetables such as broccoli, cabbage, cauliflower, and Brussels sprouts. Given the interest in these compounds as chemopreventive antileukemia agents, recent studies have examined the pharmacokinetics of sulforaphane. It has been shown that sulforaphane can be absorbed, achieve micromolar concentrations in the blood, and accumulate in tissues,[15] and in rats an estimated 20 μM SFN peak plasma concentration was achievable without causing toxicity.[34] Predicated on these studies, we chose to use the more conservative estimate in order to ensure that our observations were clinically relevant and achievable through diet alone. It should also be noted that the dose of imatinib that we employed is also clinically achievable.[35] Given the observations that the addition of sulforaphane enhanced IM-mediated antileukemia effects especially in the LSC population and the recognized safety and ease of sulforaphane administration through diet, IM/SFN combined therapy may represent a beneficial therapeutic option in the future.

In summary, our study has provided evidence that the combination of IM and SFN could target and eliminate IM-resistant LSCs. We demonstrated that IM/SFN combined treatment suppressed two major pathways, namely, BCR-ABL and β-catenin, rendering the LSCs vulnerable for ROS attacks. Since SFN has been indicated well tolerated in both animals and human, SFN might be a good candidate for adjuvant therapy for refractory CML patients.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The primers used in the RT-PCR experiments are listed in the supporting information section. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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