Sulforaphane Induces Cell Cycle Arrest and Apoptosis in Acute Lymphoblastic Leukemia Cells
Koramit Suppipat, Chun Shik Park, Ye Shen, Xiao Zhu, H. Daniel Lacorazza

Abstract

Acute lymphoblastic leukemia (ALL) is the most common hematological cancer in children. Although risk-adaptive therapy and supportive care have improved the survival of ALL patients, disease relapse is still the leading cause of cancer-related death in children. In this study, we report that purified sulforaphane, a isothiocyanate found in cruciferous vegetables, has anti-leukemic properties in a broad range of ALL cell lines and primary lymphoblasts from pre-B ALL patients. The treatment of ALL leukemic cells with sulforaphane resulted in dose-dependent apoptosis and G2/M cell cycle arrest, with the activation of caspases (3, 8, and 9), inactivation of PARP, p53-independent upregulation of p21CIP1/WAF1, and inactivation of the AKT and mTOR survival pathways in most of the tested cell lines by lowering the levels of both total proteins. Finally, the administration of sulforaphane to the ALL xenograft models resulted in a reduction of tumor burden, suggesting a potential role as an adjunctive agent to improve the therapeutic response in high-risk ALL patients with activated AKT signaling.


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Introduction

Acute lymphoblastic leukemia (ALL) is the most common hematological malignancy in children. The incidence of the two prognoses; approximately 70–80% of ALL cases are of the precursor B-cell lineage (pre-B ALL), whereas T-cell ALL (T-cell ALL) is rarer (lower incidence and worse prognosis [1]). Although risk-adaptive therapy has improved the treatment outcome of childhood ALL, 20% of the patients with ALL do not respond or fail induction treatment [2]. A poor understanding of ALL pathobiology has prevented the development of targeted therapies to treat r common cause of cancer-related deaths in children [3]. Therefore, the development of novel agents is critical to generate effective and salvage agents to treat relapsed disease.

Although relapses in pre-B ALL are frequently associated with high-risk disease (i.e., BCR-ABL translocation, hypodiploidy, and MLL rearrangement), relapses can occur within all currently defined risk groups [3], [4]. The majority of relapse standard (i.e., age 1–9 y/o and white blood cells <50,000/µl) or high-risk categories (i.e., age <1 y/o or >9 y/o, WBC >5 x 10^9/L at diagnosis; testicular disease at diagnosis) [3]. The survival rate of patients with relapsed pre-B ALL ranges from 20 to 50%, deparing...
contrast, patients with T-cell ALL who develop bone marrow relapse at any time during therapy are more difficult to treat compared to relapsed pre-B ALL patients [5], [6]. Thus, a better understanding of the pathogenesis of ALL and the ider with more aggressive disease can aid in the development of new therapeutic strategies for children with high-risk disease to proliferate and survive compared to normal cells is an important factor associated with disease severity [7]. Leukemic cell survival through the loss of key cell cycle checkpoint controls, such as CDKN2A/B misexpression, and the activation of pPI3K/AKT/mTOR pathway [8], [9], [10], [11], [12], [13]. Therefore, chemical compounds that are able to induce cell cycle selectively in leukemic cells both in vitro and in preclinical mouse models may be the next line of chemotherapeutic drugs that are ut leukemic cells.

Many standard chemotherapeutic agents have been discovered from natural sources (e.g., daunorubicin and cytarabine isothiocyanate found in cruciferous vegetables and is endowed with both preventive and therapeutic activities in solid tum in the US found that individuals who consumed a diet rich in cruciferous vegetables (i.e., broccoli and cabbage) had a low colon cancer [15], [16], [17], [18], [19]. Furthermore, the consumption of raw cruciferous vegetables inversely correlates cancer chemopreventive property has been largely attributed to the activity of isothiocyanates derived from the metabolism of cruciferous vegetables. The glucosinolate glucoraphanin is converted to SF by myrosinases that are present in cruciferous highly reactive and hydrophobic compound that can alter cellular function by entering cells where SF is either conjugated mercapturic pathway or it forms thionacetyl adducts with the thiol groups of not yet identified proteins in cancer cells. SF and has been tested in animal models of human cancer, such as T-cell leukemia, breast, colon, and prostate cancer, by (p21), p53 and Bax [21], [22], [23], [24], [25]. SF also causes G1/S and G2/M cell cycle arrest through alterations in the p21Cip1/Waf1, and the tumor suppressor KLF4 [23], [26], [27], [28], [29]. Moreover, a recent report showed that SF restric P13K/AKT signaling in the PTEN-null mouse model of prostate cancer [30].

In this study, we describe the anti-leukemic effect of SF in pre-B ALL and T-ALL cell lines and primary samples from per G2/M cell cycle arrest, higher p21 levels, cleavage of PARP and caspases (3, 8, and 9), and the inhibition of AKT/mTOR that the administration of SF controlled the expansion of leukemic cells in pre-clinical xenograft models of ALL. This study adjunctive agent to increase remission rates in high-risk ALL patients and to treat other hematological malignancies with

Materials and Methods

Ethics Statement

The Baylor College of Medicine Institutional Review Board approved the use of human samples for this study. Primary tissue repository at Texas Children's Hospital that collected samples following written informed consent. We received an Leukemia Research Interest Group at Texas Children's Hospital.

All mice were maintained in specific pathogen-free conditions at the Baylor College of Medicine (Houston, TX, USA). All approval of the Institutional Animal Care and Usage Committee of Baylor College of Medicine.

Chemicals

Synthetic DL-Sulforaphane (SF) was purchased from Sigma-Aldrich (St. Louis, MO) and LKT Laboratories (St. Paul, MN) was dissolved in dimethyl sulfoxide (DMSO) to generate a 40 mM stock concentration and stored at −20°C. SF was diluted RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The volume of DMSO was less than 0.1% of the DMSO in culture medium was used as the vehicle control. S-(N-Methylsulfinylbutylthiocarbamoyl)-glutathione(SF-glutathi S-(N-Methylsulfinylbutylthiocarbamoyl)-L-cysteine(SF-cysteine), and N-Acetyl-S-(N-Methylsulfinylbutylthiocarbamoyl)-L-c Laboratories.

Cell Lines and Cell Culture

The Nalm-6, REH and RS-4 cell lines were obtained from Dr. Karen Rabin (Texas Children's Cancer and Hematology Ce from the American Type Tissue Collection (REH, RS4) and the DMSZ German Collection of Microorganisms and Cell C lymphoblastoid cell line (LCL) was provided by Dr. Catherine Bollard (Center for Cell and Gene Therapy, Baylor College KOPTK1 T-ALL cell lines were obtained from Dr. Adolfo Ferrando (Columbia University, New York) [32] and Jurkat cells All of the cell lines were maintained in RPMI-1640 medium (Lonza) supplemented with 10% FBS, 2 mM glutamine, 1 mM streptomycin (10 µg/ml).

Patient Samples
Bone marrow or peripheral blood samples from pre-B ALL and T-ALL patients collected at the time of diagnosis were obtained from the Texas Center tissue repository (>80% blast cells). The samples were collected after written informed consent and frozen lymphoblasts were supplied in a manner. All experiments with patient samples conformed to the regulatory standards approved by the Baylor College of Medicine Institutional Review Board.

Mononuclear cells from healthy donors were isolated using Ficoll gradient from peripheral blood collected according to institutional review board procedures.

**In vitro Cell Viability Assay**

The cell lines were cultured overnight in 96-well plates at a density of 2×10^4 cells/well. The next day, SF was added (0–additional 24–48 hours. The primary patient samples were cultured for 4 hours in 96-well plates at a density of at least 2×10^24–48 hours in the presence of SF (0–40 µM). Cell viability was measured using the CellTiter-Glo Luminescent Viability assay and the number of viable cells was estimated as relative luminescent units. The half-maximal inhibitory concentration (IC50) was calculated from dose by nonlinear regression analysis using GraphPad software.

**Analysis of Apoptosis and DNA Content**

The cell lines were cultured in 6-well plates at a final concentration of 4×10^5 cells/ml and incubated overnight before the addition of SF (7.5 µM) and 7-AAD-positive cells were analyzed by flow cytometry (Biosciences). To measure DNA content, the cells were resuspended in PBS containing 0.1% sodium citrate, 0.1% Triton-X 100 and RNase A (10 µg/ml). Nuclei were stained with propidium iodide (50 µg/ml). The cell cycle distribution was analyzed by flow cytometry (FACScanto) and calculated using FlowJo software.

**Immunoblots**

The leukemic cells were harvested after 24 hours of incubation with SF (7.5 µM) or the vehicle control. The cells were lysed with a 1% Nonidet-containing 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and a cocktail of phosphatase and protease inhibitors samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes. The immunoblots were probed with antibodies (Cell Signaling): rabbit polyclonal anti-PARP, mouse monoclonal anti-caspase 3, mouse monoclonal anti-caspase 8, mouse monoclonal anti-p21, mouse monoclonal anti-p53, rabbit polyclonal anti-AKT, rabbit monoclonal anti-phospho-AKT (Ser473), rabbit monoclonal anti-phospho-mTOR, rabbit monoclonal anti-cyclin B1, rabbit monoclonal anti-phospho-cdc2 (Tyr15), rabbit anti-cdc2, rabbit anti-ph-cdc25C. Mouse anti-cyclin D1 antibody was obtained from Santa Cruz Biotechnology.

**Xenograft Mouse Models**

The Nalm-6 (pre-B ALL) cells were transduced with retrovirus generated by the co-transfection of 293T cells with the psi-amphotropic envelope. Transduced tumor cells, which were marked by GFP expression, were selected by fluorescence-activated cell sorting (FACS) and transferred to NOD/SCID mice. The cell lines were established in the presence of SF (0–40 µM) or the vehicle control. To induce subcutaneous tumors, 5×10^6 Nalm-6-FFluc cells embedded in high concentration basement membrane matrix (BD Biosciences) were subcutaneously into the flanks of NOD/SCID mice. Bioluminescent imaging was performed after one week of tumor establishment (day 0 treatment). For bioluminescence detection, the images were acquired in anesthetized mice using the Odyssey Infrared Imaging System (Xenogen) 10 minutes after intraperitoneal injection with 50 mg/kg D-luciferin. All mice were maintained in specific pathogen-free conditions at the Baylor College of Medicine (Houston, TX, USA). All experiments were performed with the approval of the Institutional Animal Care and Use Committee.

**Statistical Analysis**

Two-tailed unpaired Student’s t-tests were performed using GraphPad software. Differences were considered significant if p-values were indicated in each figure legend.

**Results**

**Sulforaphane Induces Apoptosis in Pre-B ALL and T-ALL Cells**

SF is a natural product found in cruciferous vegetables and has shown anti-cancer properties in breast, prostate, and colorectal cancer. We investigated whether purified SF is also effective against hematological malignancies by testing its activity in ALL leukemia cell lines. In vitro studies revealed that SF induced apoptosis in ALL cells, as evidenced by increased annexin-V- and 7-AAD-positive cells and decreased cell viability. Further analysis of apoptosis and DNA content using flow cytometry demonstrated activation of the apoptotic pathway in ALL cells treated with SF.

**Immunoblot Analysis**

Immunoblots were performed to assess the activation status of various downstream targets involved in the apoptotic pathway. The cell lines were treated with SF (7.5 µM) and harvested after 24 hours of incubation. Western blot analysis revealed activation of caspase-3, caspase-8, and PARP, indicating the induction of apoptosis in ALL cells treated with SF. The expression levels of pro-survival proteins such as AKT and mTOR were also analyzed and showed a decrease in their phosphorylated forms, suggesting a suppression of the PI3K/AKT/mTOR pathway.

**Xenograft Mouse Models**

Xenograft models were established to evaluate the in vivo efficacy of SF in ALL. Nalm-6 (pre-B ALL) cells were transduced with GFP and injected subcutaneously into the flanks of NOD/SCID mice. SF treatment led to a significant reduction in tumor burden, as evidenced by bioluminescent imaging and histological analysis. The results indicated that SF has the potential to induce regression of ALL xenografts in vivo.

**Statistical Analysis**

Statistical analysis was performed using GraphPad software to validate the observed effects of SF in vitro and in vivo. Two-tailed unpaired Student’s t-tests were conducted to compare the cell viability and apoptosis rates between control and SF-treated groups. The analysis confirmed the significant induction of apoptosis and anti-tumor effects of SF in ALL cells and xenografts.

**Conclusion**

Sulforaphane (SF) is a natural compound found in cruciferous vegetables that has shown anti-cancer properties in various preclinical models. Our study demonstrates the potential anti-ALL activity of SF, both in vitro and in vivo, by inducing apoptosis and inhibiting tumor growth. Further exploration of SF’s mechanism of action and clinical translation are warranted.
µM) significantly reduced the viability of the Nalm-6 human pre-B ALL cell line (Fig. 1A). We next examined the effects of the viability of the cultured pre-B ALL and T-ALL leukemic cell lines compared to the EBV-transformed B-cell lymphoblasts “non-leukemic” control. SF selectively killed the Nalm-6, REH, and RS-4 pre-B ALL cells in a dose-dependent manner (F). Non-leukemic cells with the highest concentration of SF resulted in a 20% reduction in cell viability (Fig. 1B). Similar to th and KOPTK1 T-ALL cell lines were susceptible to SF-mediated cytotoxicity, and nearly no viable leukemic cells survived. Consistent with the inhibitory concentrations reported for other cancer cells [29], [36], [37], SF exhibited a half-maximal range (Table 1). Because patient-derived cells often accumulate additional genetic aberrations during the establishment of findings using primary lymphoblasts collected at diagnosis from the pre-B ALL and T-ALL pediatric patients at the Texas Children’s Cancer and (48 hours) reduced survival of normal PBMC due to their limited proliferative and survival capacity in vitro, the IC50 (30. (p<0.001) than primary samples from pre-B ALL (13.1±8.1 µM, n = 6) and T-ALL (10.1±2.0 µM, n = 7) patients. Finally, SF leads to abrogation of its cytotoxic properties, which is particularly important for future translation into the clinic. In c Nalm-6 cells cultured in the presence of different doses of SF-glutathione, SF-cysteine or SF-N-acetyl-cysteine was significantly improved, with 1E). For example, 7.5 µM of SF-metabolites induced only 20% cytotoxicity after 48 hours of incubation compared to 80% clinical trials should aim at maintaining a therapeutic concentration of SF (active form).

Figure 1. Inhibition of acute lymphoblastic leukemic cell survival by the SF isothiocyanate.
(A) Phase contrast images of Nalm-6 cells after culture with 10 µM SF for 24 hours. The chemical structure of sulforaphane (Nalm-6, REH and RS-4), non-leukemic LCL cells, and T-ALL cells (Jurkat, RPMI, DND41, and KOPTK1) were incubated with the specified SF concentrations for 48 hours. Each patient sample was processed in triplicate (mean SF (24 h) on PBMC freshly isolated from healthy donors (n = 4, each measured in triplicates) was compared to cell samples (mean ± S.D.). (E) Nalm-6 cells were cultured in the presence of SF-glutathione, SF-NAC, SF-cysteine, or SF (24 h) on PBMC freshly isolated from healthy donors (n = 4, each measured in triplicates) was compared to cell samples (mean ± S.D.). (n = 3). doi:10.1371/journal.pone.0051251.g001
Table 1. IC50 values in leukemic cell lines, primary lymphoblast cells from pre-B ALL and T-ALL patients.
doi:10.1371/journal.pone.0051251.t001

<table>
<thead>
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<th>Cells</th>
<th>IC50 (μM) (24 h)</th>
<th>IC50 (μM) (48 h)</th>
</tr>
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<tr>
<td>LCL</td>
<td>8.10 (26.80-243.60)</td>
<td>30.83 (17.67-33.90)</td>
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<td>Nalm-6</td>
<td>1.02 (0.66-1.88)</td>
<td>4.58 (4.37-4.79)</td>
</tr>
<tr>
<td>REH</td>
<td>10.26 (9.49-11.20)</td>
<td>4.38 (4.10-4.67)</td>
</tr>
<tr>
<td>RS-4</td>
<td>9.86 (0.35-10.41)</td>
<td>3.85 (3.69-4.01)</td>
</tr>
<tr>
<td>Jurkat</td>
<td>4.72 (4.19-5.25)</td>
<td>2.59 (1.44-3.34)</td>
</tr>
<tr>
<td>RPMI</td>
<td>7.87 (6.40-9.14)</td>
<td>5.72 (4.12-7.32)</td>
</tr>
<tr>
<td>DND41</td>
<td>13.49 (12.84-14.15)</td>
<td>7.14 (6.27-6.01)</td>
</tr>
<tr>
<td>KOPTK1</td>
<td>3.04 (2.16-3.92)</td>
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<td>10.98 (5.94-12.13)</td>
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<td>25.36 (22.43-28.68)</td>
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<tr>
<td>preB-6</td>
<td>331.2 (211-519.5)</td>
<td>20.81 (22.59)</td>
</tr>
<tr>
<td>T-1</td>
<td>51.52 (38.64-69.77)</td>
<td>11.64 (10.87-12.47)</td>
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<td>T-2</td>
<td>20.28 (18.48-22.24)</td>
<td>9.55 (8.31-11.0)</td>
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<tr>
<td>T-3</td>
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<td>13.02 (5.11-18.63)</td>
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<td>T-4</td>
<td>22.45 (19.72-25.58)</td>
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</tr>
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<td>T-5</td>
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<td>T-7</td>
<td>21.32 (19.52-23.28)</td>
<td>8.01 (6.92-9.24)</td>
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</table>

*The IC50 values were obtained by triplicate from each sample (pre-B and T-ALL). The values in parentheses represent the 95% confidence interval.
doi:10.1371/journal.pone.0051251.t001

The reduction of cell viability indicates that SF induced cell death in leukemic cells. To test this hypothesis, we incubated hours and then stained the cells with annexin-V and 7-AAD for flow cytometric analysis. In contrast to the LCL cells, SF KOPTK1 cells (Fig. 2A,B). Selectivity for leukemic cells is a desired property of chemotherapeutic agents. Next, we analyzed the effect of SF on caspases to elucidate the mechanism of SF-induced cell death in ALL cells. Pro-apoptotic signals activate caspases 8 and 9, which lead to the effector caspase 3. No significant cleavage of caspases was observed in LCL cells treated with SF (Fig. 2C). In contrast, the pre-B ALL and T-ALL showed cleavage of caspases 3, 8, and 9 (Fig. 2C). The inactivating cleavage of the DNA repair enzyme PARP was also observed (Fig. 2C).
Figure 2. SF induces apoptosis selectively in ALL cell lines.
(A) Apoptosis was evaluated by annexin-V and 7-AAD staining of LCL, Nalm-6, Jurkat and KOPTK1 cells cultured in the presence or absence of SF. The data are representative of three independent experiments. (B) The percentages of annexin-V-positive cells were determined for each cell line in the presence or absence of SF. (C) SF activates the proteolytic cascade of caspases and PARP in leukemic cells. LCL (Nalm-6, REH, and RS-4), and T-ALL cells (Jurkat, RPMI, DND41, and KOPTK1) were incubated with 7.5 µM SF for 24 hours and analyzed by immunoblotting. The arrows indicate the cleaved forms of the caspases and PARP. β-actin was used as a loading control. The data represent the mean and standard deviation (n = 3). *** P<0.001 (two-tailed Student’s t-test).
doi:10.1371/journal.pone.0051251.g002

Sulforaphane Induces G2/M Cell-cycle Arrest and Inhibits AKT-mediated Survival Signaling

Chemical compounds can induce cytotoxicity by causing aberrations in the cell cycle checkpoints, thus forcing cells to undergo apoptosis. To examine the effect of SF on the cell cycle, we examined the DNA content of LCL, Nalm-6, Jurkat and KOPTK1 cells incubated with 7.5 µM SF for 24 hours. SF-treated ALL cells accumulated in the G2/M and sub-G1 phases of the cell cycle, while LCL cells showed lower percentages in the G2/M and S phases (Fig. 3A). The aggregate analysis clearly demonstrates that the leukemic cells treated with SF accumulated in G2/M and sub-G1 phases, concurrent with the reduction in the G0/G1 and S phases (Fig. 3B).

Figure 3. SF causes G2/M cell cycle arrest in leukemic cells.
(A) The DNA content of LCL, Nalm-6, Jurkat and KOPTK1 cells incubated with 7.5 µM SF or vehicle for 24 hours was analyzed by propidium iodide staining and flow cytometry. A representative profile is shown. (B) The cell cycle distribution was calculated as described in the Materials and Methods section. The data represent the mean and standard deviation (n = 3). * P<0.05, ** P<0.01, *** P<0.001 (two-tailed Student’s t-test).
doi:10.1371/journal.pone.0051251.g003
To gain further insight into the mechanism of SF, we determined the expression of cell cycle regulators and pro-survival signals. (Nalm-6, REH, RS-4), T-ALL cells (Jurkat, RPMI, DND41 and KOPTK1), and control LCL cells were incubated for 24 hr and then analyzed by immunoblotting. We measured the expression of the tumor suppressor KLF4 and the cell cycle regulator p21 in Caco-2 cells [29]. Consistent with a previous report [38], SF increased the levels of KLF4 in all cell lines, except the Jurkat and KOPTK1 cells (Fig. 4A). In LCL cells, p21 expression was associated with the inhibition of cell growth (Figs. 3, 4). The low levels of KLF4 expression observed in ALL cells, regardless of SF incubation, suggests that KLF4 activity (Fig. 4A). Epigenetic silencing of the KLF4 gene, an alternate mechanism that may be responsible for the low expression of KLF4 supported by the 8-fold (KOPTK1), 13-fold (RPMI), 6-fold (DND41), and 5-fold (Jurkat) increases of KLF4 expression upon culture of the leukemic cell lines with 5-azacytidine (not shown). In accordance with our findings, KLF4 gene was found hypermethylated in adult T-ALL cells because SF can induce G1 cell cycle arrest by inhibiting expression of Cyclin D1 in human colon carcinoma HT-29 cells [40].

Figure 4. Effect of SF on the levels of total and phosphorylated proteins involved in the G2/M cell cycle arrest.

(A) The protein levels of KLF4, p21, p53, cyclin B1, cyclin D1, cdc2, and cdc25C and phosphorylation of cdc2 (Tyr 15) and cdc25C (Ser 216) were assessed by immunoblotting in LCL, pre-B ALL, and T-ALL cell lines incubated with 7.5 µM SF or vehicle for 24 hours. β-actin was used as a loading control representative of three independent experiments. (B) Detection of phospho-H2AX (pH2AX) in nuclei by flow cytometry in Nalm-6 and Jurkat cells treated with 7.5 µM SF or Etoposide (ETO) for 24 hours. The data are representative of three independent experiments. (C) Diagram depicting the G2/M cell cycle arrest of ALL cells.

doi:10.1371/journal.pone.0051251.g004

To further study the mechanism of cell cycle arrest, we first analyzed the levels of Cyclin B1 in SF-treated ALL cell lines. It has been reported that SF induces G2/M arrest by altering the levels of Cyclin B1 in PC-3 prostate cancer cells [40]. In acute lymphoblastic leukemia, SF increased the expression of Cyclin B1 in the REH, RS-4 and T-ALL cell lines (levels of phospho-Cdc2 (Tyr-15) in ALL cells treated with SF, suggesting that SF inhibits the Cdc2/Cyclin B1 complex by (inactive) before elevated levels of Cyclin B1 (Fig. 4A). Cdc25C activates Cdc2/Cyclin B1 complex by dephosphorylation at Ser 216 in response to DNA damage [41], [42]. Since we did not detect significant changes in total levels of SF likely activates the Wee1 or Myt1 kinases that phosphorylate Cdc2 [43], [44], in addition to the inhibitory role of whether SF causes DNA damage, we determined by flow cytometry nuclear expression of phospho-H2AX, a sensitive marker of double-strand DNA breaks (DSB). Nalm-6 cells treated with 7.5 µM SF showed considerably lower phospho-H2AX content compared to control Nalm-6 cells treated with etoposide (topoisomerase inhibitor) as a positive control. Nalm-6 cells treated with 7.5 µM SF showed considerably lower phospho-H2AX content compared to control Nalm-6 cells treated with etoposide, while almost no positive cells were detected in Jurkat cells (Fig. 4B). Collectively, we inactivated Cdc2 and elevating p21 levels without inducing significant DNA damage (Fig. 4C).

Finally, we were interested to study the effect of SF on survival signals, as many patients with T-ALL exhibit activation of the AKT and mTOR pathways by lowering the protein levels of both total and phosphorylated AKT and mTOR (Fig. 5). In conclusion, these results suggest that SF induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells by targeting key molecules involved in cell cycle regulation and survival pathways.
the LCL cells showed a slight activation of AKT signaling in response to SF treatment. Collectively, these findings indicate survival signals in leukemic cells.

<table>
<thead>
<tr>
<th></th>
<th>Pre-B ALL</th>
<th>LCL</th>
<th>Nalm-6</th>
<th>REH</th>
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<td>-</td>
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<td>β-Actin</td>
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</table>

Figure 5. SF inhibits the AKT/mTOR survival pathway in leukemic cells.

The protein levels of phosphorylated and total AKT and mTOR were examined by immunoblotting. LCL, pre-B ALL, or 7.5 µM SF or vehicle for 24 hours. β-actin was used as a loading control. The data are representative of three independent experiments.

Pre-clinical Testing of SF Using Xenograft Leukemia Models

First, we tested the efficacy of SF in controlling the expansion of Nalm-6 cells injected in NOD/SCID mice to model systemic disease detected by bioluminescence in the first week whereas in the peripheral blood were detected by flow cytometry at a later stage, when mice showed disease (weight loss and hind-limb paralysis). The NOD/SCID mice injected with Nalm-6-FFluc cells were randomized to two groups: the experimental group received daily administration of SF (2 mg, i.p.), and the control group received vehicle starting 24 hours after injection of leukemic cells. The tumor-bearing mice showed that treatment with SF reduced disease progression in the first 2 weeks, although we did not observe significant survival (Fig. 6A). The partial response may be due to the low SF concentration in tissues, such as the bone and spine, expand during the first few weeks. Therefore, we next tested the oral administration of SF in pre-established subcutaneous xenograft tumors. Mice treated with SF showed a 4-6-fold increase in tumor burden compared to the tumor size before treatment (day 0). In contrast, tumor reduction in growth after 4 days of treatment (Fig. 6B). Our findings demonstrate the effectiveness of SF in controlling hematological malignancies.

Figure 6. SF inhibits leukemia progression in xenograft models.

The low toxicity associated with the administration of extracts enriched in SF in human clinical trials supports further studies on the role of SF in hematological malignancies.
Figure 6. *In vivo* anti-leukemic effect of SF in ALL xenograft models.

(A) Nalm-6 cells were transduced with the FFLuc retrovirus and injected intravenously into NOD/SCID mice. Twenty-four hours later, mice with SF or vehicle (2 mg i.p. daily) and monitored weekly for the distribution of leukemic cells by bioluminescence imaging (BLI). (B) The Nalm-6 were mixed with a high-protein matrigel and injected into the flank of NOD/SCID mice. One week later, tumor establishment was confirmed mice were treated by oral gavage (2 mg/gave, twice daily) for 7 days. The total counts were determined for the control- and SF-treated mice representative of three independent experiments. The statistical significance was calculated using the two-tailed Student’s doi:10.1371/journal.pone.0051251.g006

Discussion

There is emerging evidence that SF has anti-carcinogenic properties in addition to the chemopreventive effect of broccoli investigated in solid tumors. The main goal of this study was to test the anti-leukemic properties of SF in the most prevalent hematological cancer because the development of new chemodrugs for pediatric patients has lagged behind the development of new therapies for adult leukemia. In that synthesized DL-Sulforaphane induced a dose-dependent cytotoxicity in ALL cell lines and primary lymphoblasts from ALL or T-ALL. Most importantly, normal peripheral blood mononuclear cells were more resistant than patient lymphoblasts

Based on previous reports indicating that SF-mediated cytotoxicity in Caco-2 cells was associated with the increased expression of the cyclin-dependent kinase inhibitor p21, we initially hypothesized that SF would also induce KLF4 expression in ALL cells observed in ALL cell lines suggested an epigenetic silencing that could not be reversed by SF; in support of this hypothesis in T-ALL cell lines cultured with a demethylating agent. Despite the low expression levels of KLF4 and p53, we observe ALL and T-ALL cells following SF treatment. Because the anti-tumor activity of many conventional chemotherapeutic agents (e.g., etoposide) is mediated by p53 [45], [46], the p53-independent activation of p21 that was observed upon SF treatment approach for ALL patients that fail to respond to conventional agents targeting p53. In addition to p21 upregulation, SF by promoting phosphorylation of Cdc2 in spite of increased levels of Cyclin B1. Our findings are consistent with previous G2/M cell cycle arrest by regulating cell cycle proteins [26], [27], [28], [29], [47].

Several reports have shown activation of the mitochondrial or death receptor pathways in cancer cells cultured with SF showed that SF induced apoptosis in pre-B ALL (Nalm-6, REH, and RS-4) and T-ALL (Jurkat, RPMI, DND41, and KOP) and 9 and inactivating PARP. Interestingly, SF-mediated cytotoxicity appeared specific to leukemic cells, as no significant change was observed in the non-leukemic control cells. In addition to cell cycle arrest and apoptosis, SF inhibited the expression of both the total and phosphorylated forms of AKT and mTOR. This result was of interest because the A choice in the development of alternative ALL therapies since approximately 30% of pre-B ALL patients display mutations including RAS, PTPN11 and FLT3 [49], [50], [51], [52]. In addition, a gene expression study showed that AKT activation glucocorticoids in pre-B ALL [10]. Inactivation of PTEN by deletion, mutation, epigenetic silencing, or post-translational inactivation of AKT also contributes to the development of T-ALL [13], [53] [54]. Therefore, inhibition of AKT pathways may be effective in the treatment of ALL patients with activated AKT pathways.

Several therapeutic compounds that target the cell cycle and the PI3K/AKT/MTOR pathway are being tested as ALL the nelarabine are cell-cycle dependent CDK inhibitors that have been recently approved to treat pediatric ALL [55]. Alterna spectra of activity are in clinical development as combination therapies to eliminate drug-resistant ALL, such as the dual PI3K/PDK-1 inhibitor BAG956 [56], [57]. The PI3K-AKT inhibitor Ly294002 has also shown marked activity in leukemia to perturbing the cell cycle, SF also inhibited the AKT survival pathway by lowering protein levels of total and phosphoryl
advantages to using natural food products for cancer treatment such as correct chirality to bind biomolecules, safety, or clinical trials. SF also induced phase 2 metabolic enzymes (detoxification) in addition to its anti-carcinogenic properties [activities as a chemotherapeutic agent by inducing cell death in cancer cells and reducing toxicity in multi-drug intensific

The pharmacokinetics of SF have been extensively studied in humans. After oral intake of broccoli, SF reaches a transient µM [60], [61]. The bioavailability of SF depends on food processing, as cooked broccoli demonstrated lower serum concentrations in the micromolar range (1–7 µM in ALL cell lines and 5–20 µM in primary lymphoblastic cells) than SF cannot be reached through the dietary consumption of cruciferous vegetables. We tested the efficacy of SF in two proof-of-principle that SF can inhibit the expansion of leukemic cells in vivo. Intraperitoneal administration of SF to NOD-scid mice expanded of leukemic cells in the first two weeks. The inability to efficiently eliminate or control the expansion of leukemic concentrations achieved in leukemic cell niches such as the bone marrow. However, oral administration of SF led to a significant reduction in the expansion of leukemic cells in a model for pre-established lymphoma tumors. Collectively, our preclinical studies showed that SF can live, which supports future clinical trials as an adjunctive chemotherapy in ALL.

In summary, the results presented in this study demonstrate the anti-leukemic properties of SF in acute lymphoblastic leukemia. Furthermore, we showed that SF inhibits the AKT/mTOR survival pathway. This is the first report on the anti-malignancies, supporting its use as an adjunctive chemotherapy in ALL.

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Author Contributions

Performed the experiments: KS CSP XZ YS. Analyzed the data: KS HDL CSP. Wrote the paper: KS CSP HDL.

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