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# BMI1 Inhibitors Down-regulate NOTCH Signaling and Suppress Proliferation of Acute Leukemia Cells

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**Abstract.** *Background/Aim: B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) is up-regulated in several cancers; therefore, we investigated the effects of BMI1 inhibitors on leukemia cells. Materials and Methods: Four acute myeloid leukemia and two T-lymphoblastic leukemia cell lines were treated with BMI1 inhibitors artemisinin, PRT4165, and PTC-209 and analyzed for cell proliferation and gene expression by microarray and immunoblotting. Results: PTC-209 and PRT4165 suppressed the growth of all cell lines through apoptosis.Artemisinin acted only on Jurkat cells. BMI1 inhibitors and BMI1-specific siRNA down-regulated the expression of NOTCH signaling proteins NOTCH1, HES1, and MYC. All but one cell lines did not have the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene targeted by BMI1, thus the inhibitors acted through CDKN2A-independent pathways. Conclusion: BMI1 inhibition suppressed proliferation of leukemia cells through NOTCH signaling which functions downstream of BMI1, suggesting that BMI1 inhibitors can be candidate targeted drugs against leukemia.*

Polycomb group (PcG) proteins are transcriptional repressors playing key roles in self-renewal of stem cells through chromatin remodelling. PcG proteins function within two multiprotein complexes called polycomb repressive complex (PRC) 1 and PRC2, which regulate different steps of gene expression. PRC2 binds to the target genes and trimethylates histone H3 lysine 27 (H3K27me3) in chromatin (1), whereas PRC1 recognizes H3K27me3, and E3 ubiquitin ligase RING1A/B within PRC1 ubiquitinates histone H2A at lysine 119 (2). As a result, concerted activity of PRC1 and PRC2 leads to silencing of target genes linked to tumour development (3).

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B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) is a key regulatory component of PRC1 (4) known to promote cell proliferation by inhibiting expression of various genes such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*) which encodes CDK inhibitor $p16^{INK4A}$ and tumour suppressor $p14^{ARF}$. BMI1 was shown to be up-regulated in several cancers, including acute leukemia (4–6), and a novel small molecule inhibitor of BMI1, PTC-209, has demonstrated inhibitory activity in human colorectal cancer (7), acute leukemia (6), and myeloma (8).

In this study, we investigated the role of BMI1 in leukemia cell growth using three BMI1 inhibitors, artemisinin, PTC-209, and PRT4165 and six leukemia cell lines, THP-1, TMD7, OCI/AML2, OCI/AML5, Jurkat and KOPT-K1. We also examined the underlying molecular mechanisms, focusing on NOTCH signaling which is crucial for self-renewal of leukemia stem cells (9), and analyzed the expression of NOTCH, cleaved NOTCH, the hairy and enhancer of split 1 (HES1), and v-myc myelocytomatosis viral oncogene homolog (MYC).

## Materials and Methods

*Cell lines and BMI1 inhibitors.* We used six leukemia cell lines (THP-1, TMD7, OCI/AML772, and OCI/AML5 derived from AML, and Jurkat and KOPT-K1 derived from T-ALL) and normal lymphocytes from two healthy volunteers who provided informed consent. THP-1 was obtained from the Health Science Research Resource Bank (Osaka, Japan), TMD7 was established in our laboratory (10), and OCI/AML2 and OCI/AML5 were established at the Ontario Cancer Institute (11). Jurkat cells were purchased from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK), and KOPT-K1 was donated by Drs. Harashima and Orita, Fujisaki Cell Center (Okayama, Japan). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum in a humidified 5%-CO2 atmosphere. BMI1 inhibitors artemisinin, PTC-209, and PRT4165 were purchased from Sigma (St. Louis, MO, USA), Xcess Biosciences Inc. (San Diego, CA, USA), and Axon Medchem (Groningen, The Netherlands), respectively, and dissolved in dimethyl sulfoxide (DMSO). The inhibitors were chosen based on previous results showing that artemisinin and PTC-209 suppressed *BMI1* mRNA expression (7, 12) and PRT4165 inhibited BMI1 by down-regulating BMI1/RING1A self-ubiquitination (12, 13).

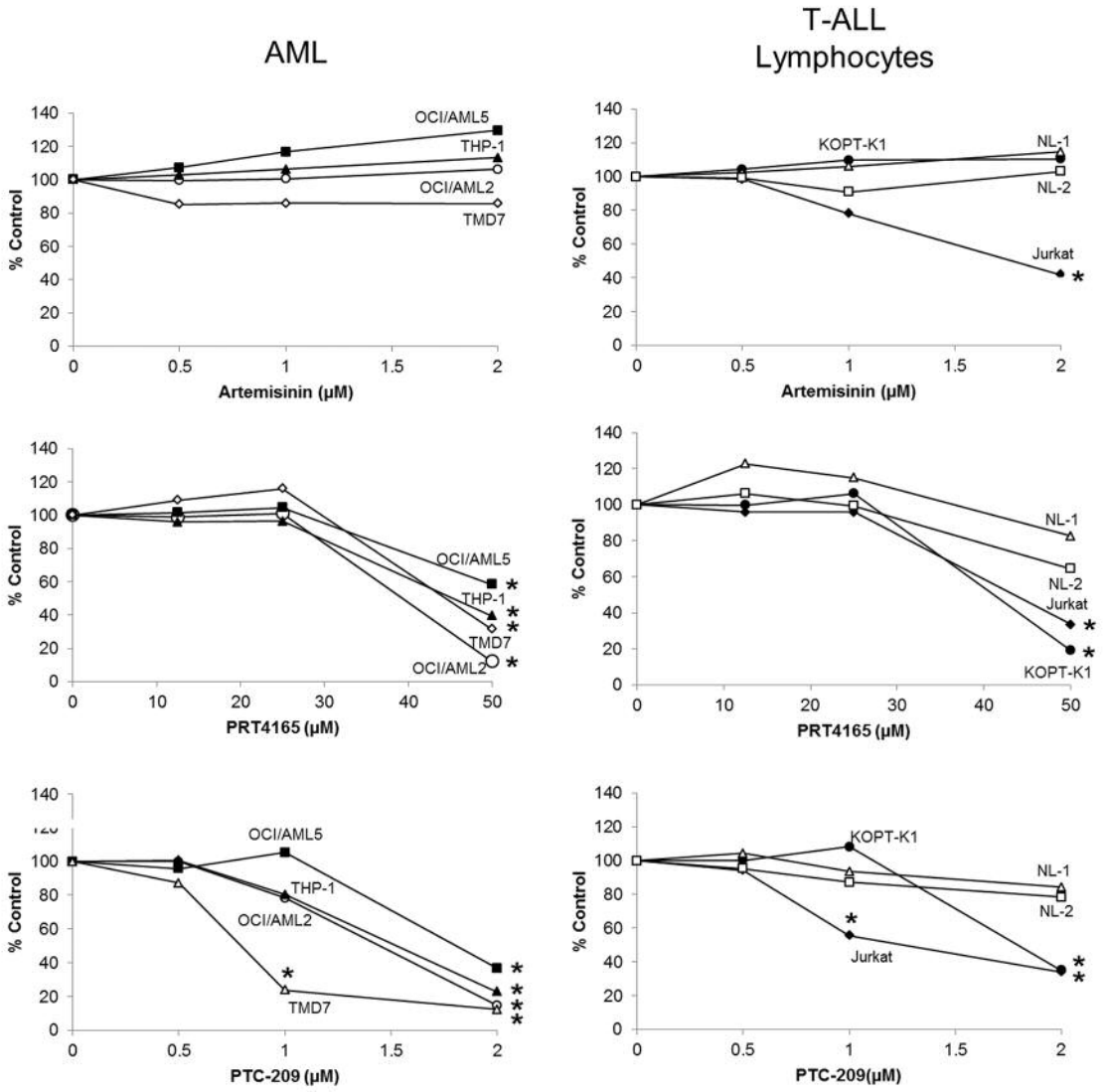


Figure 1. *Effects of BMI1 inhibitors on cell growth. Cells were cultured with the indicated concentrations of inhibitors for three days and cell growth was evaluated using a colorimetric assay. The results are expressed as a percentage of the mean OD in inhibitor-treated cells normalized to that in control (DMSO-treated) cells. NL: Normal lymphocytes. * p<0.05 compared to control.*

*Cell growth assay*. Cell proliferation was assessed using the colorimetric WST-8 assay (Dojindo Laboratories, Kumamoto, Japan). Cells were cultured with or without increasing concentrations of the inhibitors in 96-well culture plates for three days. Then, WST-8 and 1-methoxy-5-methylphenazinium methyl sulphate were added, and optical density (OD) was measured using an enzyme-linked immunosorbent assay plate reader. Relative cell proliferation was calculated as the percentage of the mean OD value normalized to that of the control. The effects of the inhibitors on cell morphology and apoptosis were examined in cytospin preparations stained with Wright's stain and observed under a microscope.

*Apoptosis assay and flow cytometry*. Cells treated with BMI1 inhibitors were stained with Annexin V-FITC and propidium iodide, and analysed using a FACS Calibur cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to evaluate the induction of apoptosis.

*Western blotting*. Protein expression was examined by immunoblotting. After culturing with the inhibitors, cells were harvested and lysed. The lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies against BMI1, NOTCH1, cleaved NOTCH1 (Val1744), HES1, MYC, caspase-3 (Cell Signaling Technology, Danvers, MA, USA), $p16^{INK4A}$, $p14^{ARF}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and α-tubulin (Abcam, Cambridge, MA, USA) which was used as a loading control. Immunoreactive bands were detected using an Pierce Enhanced Chemiluminescent Western Blotting kit (Pierce Biotechnology,

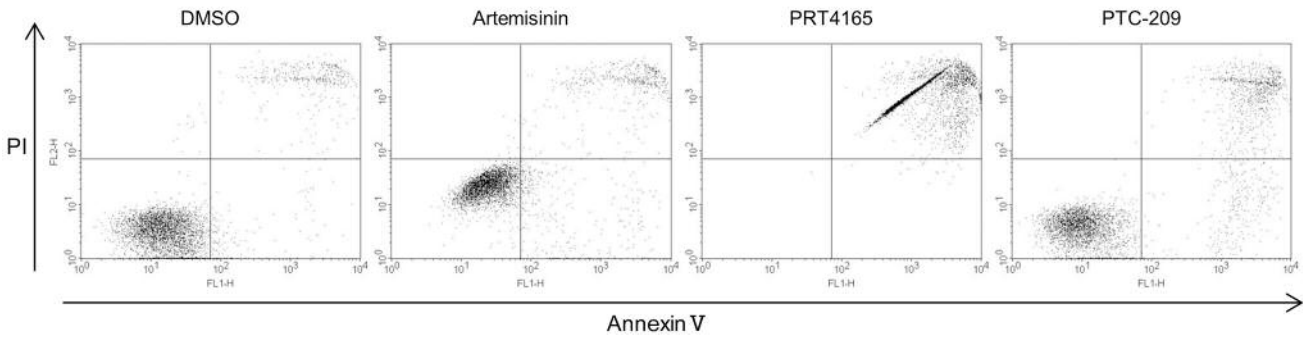


Figure 2. *Apoptosis assay of Jurkat cells treated with BMI1 inhibitors. Cells were cultured with 2 μM artemisinin, 50 μM PRT4165, and 2 μM PTC-209 for 48 h, stained with Annexin V-FITC and propidium iodide (PI), and analysed for apoptosis by flow cytometry.*

Rockford, IL, USA). Each assay was repeated more than twice to ascertain reproducibility.

*BMI1 knockdown by siRNA*. To confirm specificity of the effects exerted by BMI1 inhibitors, we performed *BMI1* knockdown by small interfering RNA (siRNA). Three different pre-designed siRNAs (Stealth siRNA™) targeting *BMI1* (HSS 101038, 101039, and 101040) were purchased from Life Technologies (Carlsbad, CA, USA); stealth RNAi negative control Duplex was used as a control. Cells were transfected with 40 nM of each siRNA using the Neon™ pipette tip chamber-based electroporation system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and immediately transferred to culture medium.

*Microarray analysis*. To assess transcriptional effects of BMI1 inhibitors, changes in mRNA expression were examined by microarray analysis. Jurkat and THP-1 cells were treated with the inhibitors, DMSO, BMI1 siRNA, or control siRNA for 24 h. Total RNA was extracted using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany) and used to prepare cyanine-3-labeled cRNA which was hybridized to a SurePrint G3 Human GE microarray 8×60K v3 (Agilent Technologies, Santa Clara, CA, USA). The expression profile was analysed using the Agilent Feature Extraction 11.5.1.1 software.

## Results

*Effects of BMI1 inhibitors on cell growth and apoptosis*. Dose-response curves (Figure 1) showed that artemisinin suppressed proliferation of Jurkat cells, but not of the other cell lines; however, PTC-209 and PRT4165 suppressed growth of all six cell lines. At the same time, none of the BMI1 inhibitors affected the viability of normal lymphocytes in the range of concentrations used.

Analysis of cytospin preparations indicated that the BMI1 inhibitors did not alter cell morphology. However, apoptotic cells with nuclear condensation and apoptotic bodies were detected in cell lines susceptible to the inhibitors (data not shown). Consistent with these results, FACS assay revealed that BMI1 inhibitors induced apoptosis, which corresponded to cell growth retardation (representative data are shown for Jurkat cells in Figure 2).

*Effects of BMI1 inhibitors on signaling proteins*. Immunoblotting analysis confirmed BMI1 protein expression in all six cell lines (Figure 3). In addition, TMD7 cells also expressed $p16^{INK4A}$ (Figure 3) and $p14^{ARF}$ (data not shown), which were not detected in the other cell lines. Treatment with the inhibitors affected the expression and activity of signaling proteins belonging to the NOTCH pathway (Figure 3 and Table I).

Artemisinin down-regulated the expression of BMI1, NOTCH1, and cleaved NOTCH1 along with the downstream targets of NOTCH, HES1 and MYC, in Jurkat cells.

PRT4165 suppressed the expression of NOTCH1 in KOPT-K1, OCI/AML2, and OCI/AML5 cells. PRT4165 also suppressed the expression of cleaved NOTCH1, MYC, and HES1 in KOPT-K1 cells. OCI/AML2 and OCI/AML5 cells did not express cleaved NOTCH1 and HES1. PRT4165 induced cleavage of caspase-3 in five cell lines except Jurkat cells.

PTC-209 reduced the expression of BMI1 as well as NOTCH1 and MYC in all cell lines. PTC-209 induced caspase-3 cleavage in TMD7 and OCI/AML5 cells.

*Effects of BMI1 knockdown by siRNA on protein expression*. As HSS 101038 (5'-UCCUCAUCCACAGUUUCCUC ACAUU-3') was the most potent among the examined *BMI1*-specific siRNAs, its effects on protein expression are shown in Figure 4. In Jurkat cells, *BMI1* knockdown decreased the expression of NOTCH1, HES1, and MYC, which is consistent with the effects caused by PTC-209, suggesting that the inhibitors down-regulated NOTCH signaling proteins through BMI1.

*Microarray analysis*. In microarray gene expression analysis, we focused on NOTCH signaling genes. Table II shows mRNA log2 ratios between inhibitor-treated and

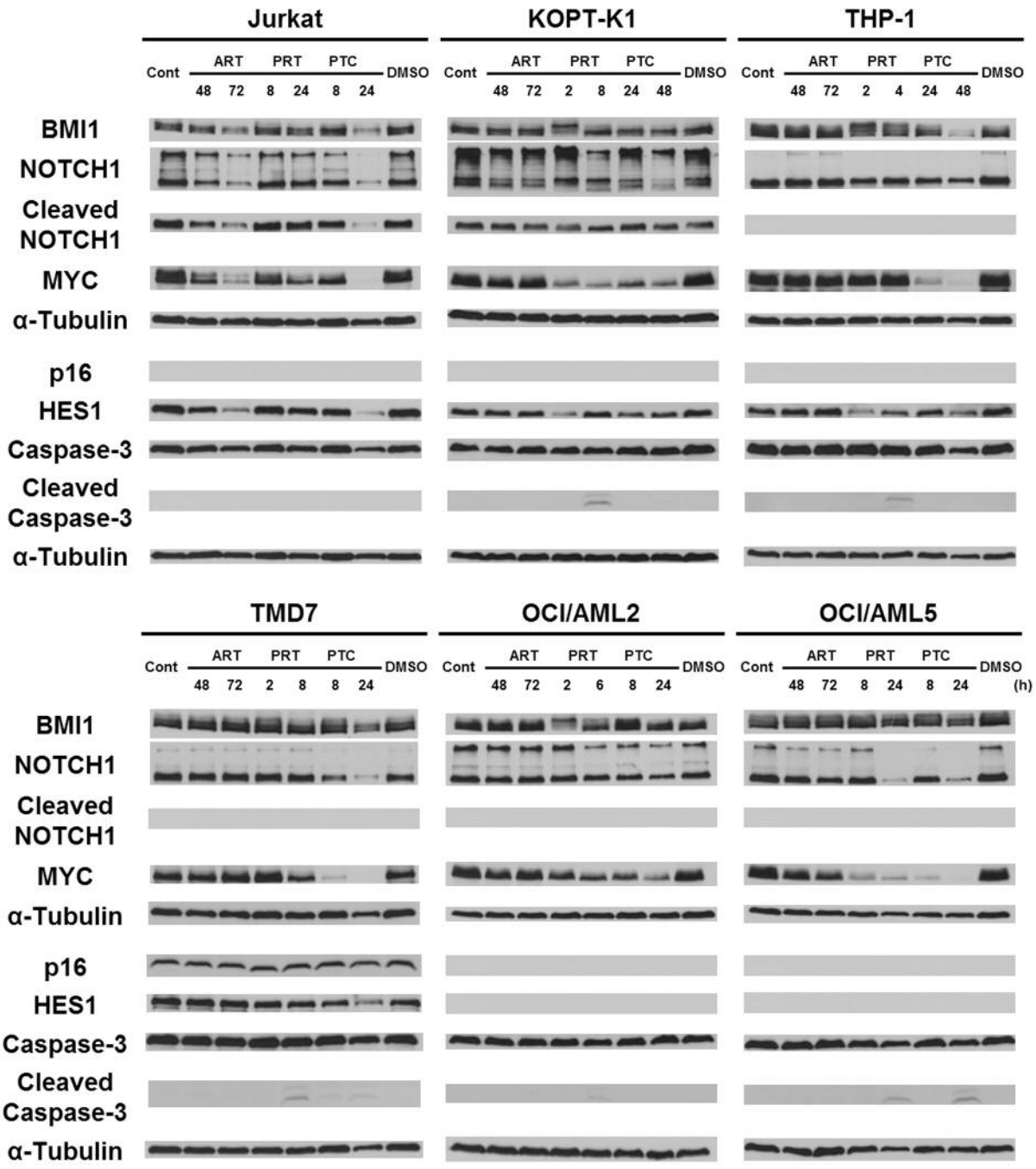


Figure 3. *Expression of BMI1, p16$^{INK4A}$, and NOTCH signaling proteins in leukemia cells treated with BMI1 inhibitors. Cells were cultured with 2 μM artemisinin (ART), 50 μM PRT4165 (PRT), and 2 μM PTC-209 (PTC) for the indicated times and analysed for protein expression by immunoblotting. DMSO was used as a vehicle control and α-tubulin as a loading control.*

DMSO-treated cells, and between *BMI1* siRNA- and control siRNA-transfected cells. Both BMI1 inhibitors and *BMI1*-specific siRNA tended to reduce the expression of *HES1* and *MYC*. *DTX1* gene encoding Deltex E3 ubiquitin ligase 1 which antagonizes NOTCH signaling (18) tended to be also down-regulated in Jurkat cells. The limitation of these results is that changes over a longer period of time are not known as the analysis was performed only in cells treated for 24 h where there was a relatively small change in gene expression.

Table I. *Summarized results of expression of BMI1 and NOTCH signaling proteins in leukemia cells treated with BMI1 inhibitors shown in Figure 3.*

| | | Jurkat | KOPT-K1 | THP-1 | TMD7 | OCI/AML2 | OCI/AML5 |
|---|---|---|---|---|---|---|---|
| ART | BMI1 | ↓ | → | → | → | → | → |
| | NOTCH1 | ↓ | → | → | → | → | → |
| | MYC/HES1 | ↓ / ↓ | → / → | → / → | → / → | → / - | → / - |
| | Cleaved-Casp3 | - | - | - | - | - | - |
| PRT | BMI1 | → | → | → | → | → | → |
| | NOTCH1 | → | ↓ | → | → | ↓ | ↓ |
| | MYC/HES1 | ↓ / → | ↓ / ↓ | → / ↓ | ↓ / - | ↓ / - | ↓ / - |
| | Cleaved-Casp3 | - | ↑ | ↑ | ↑ | ↑ | ↑ |
| PTC | BMI1 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| | NOTCH1 | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| | MYC/HES1 | ↓ / ↓ | ↓ / ↓ | ↓ / ↓ | ↓ / ↓ | ↓ / - | ↓ / - |
| | Cleaved-Casp3 | - | - | - | ↑ | - | ↑ |

Upwards arrows, downwards arrows, rightwards arrows, and minus signs indicate up-regulation, down-regulation, no significant change, and no expression, respectively.

## Discussion

We showed that BMI1 is expressed in leukemia cell lines and that BMI1 inhibitors slowed cell proliferation through induction of apoptosis, suggesting that BMI1 is involved in leukemia cell growth. It is well known that the major target of BMI1 is the *CDKN2A* gene encoding $p16^{INK4A}$ and $p14^{ARF}$ (5). However, in five of the examined cell lines, the *CDKN2A* gene was deleted, suggesting that BMI1 inhibitors exerted their effects not through the restoration of $p16^{INK4A}$ and $p14^{ARF}$ expression. This notion was also supported by the finding that in $p16^{INK4A}$-expressing TMD7 cells, the inhibitors did not up-regulate $p16^{INK4A}$.

Recently, it was reported that several molecules such as cyclin G2 are targeted by BMI1 independently from $p16^{INK4A}$ and $p14^{ARF}$ (15). In this study, we examined NOTCH signaling known to affect BMI1 (16). We found that BMI1 inhibitors reduced protein expression of NOTCH-regulated proteins such as NOTCH1, cleaved NOTCH1, HES1, and MYC not only in T-ALL cells but also in AML cells. Our results indicated that this down-regulation is not due to the off-target effects of BMI1 inhibitors, because *BMI1* knockdown by siRNA also suppressed NOTCH1 signaling. These findings suggest that the NOTCH pathway acts downstream of BMI1 (Figure 5).

These findings are in contrast to previous reports on the interaction between BMI1 and NOTCH, showing that NOTCH activation up-regulates BMI1 expression in T cells and intestinal stem cells, *i.e.*, that NOTCH acts upstream of BMI1 (16, 17). To the best of our knowledge, this is the first study to show that BMI1 regulates NOTCH signaling. Given that, the NOTCH pathway is crucial for the growth of T-ALL cells (8), our findings suggest that NOTCH down-regulation

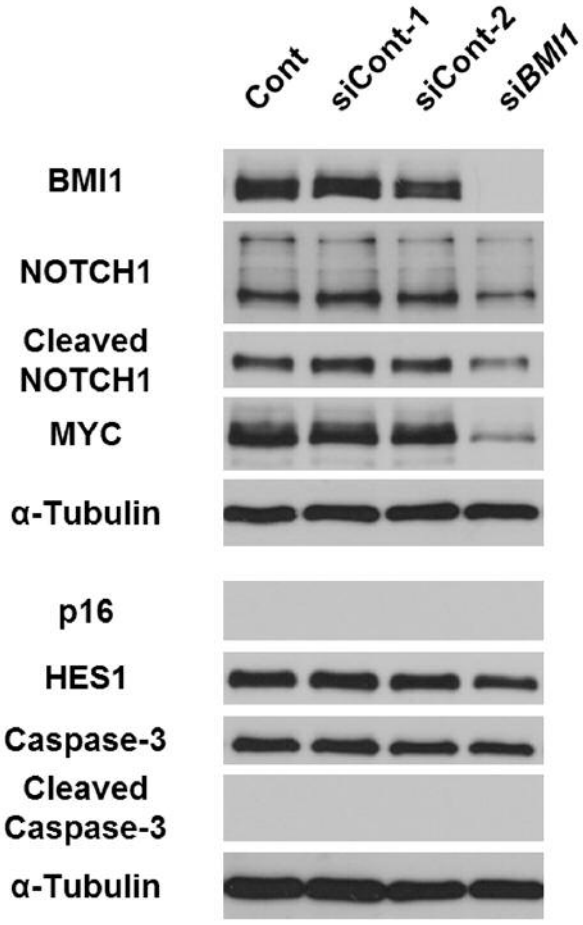


Figure 4. *Effects of BMI1 knockdown by siRNA on the expression of BMI1 and NOTCH-related proteins. Jurkat cells were transfected with BMI1 siRNA (siBMI1) and two types of control siRNA (siCont) and analysed for the expression of the indicated proteins after 48 h.*

Table II. *Effects of BMI1 inhibitors and BMI1 siRNA on the expression of representative NOTCH-related genes examined by microarrays.*

| Genes | Jurkat | | | THP-1 | | |
|---|---|---|---|---|---|---|
| | ART | PTC | siRNA | PRT | PTC | siRNA |
| *NOTCH1* | –0.19 | –0.57 | –0.26 | –0.39 | –0.41 | 0.01 |
| *HES1* | –0.48 | –2.41 | –1.01 | –1.22 | –0.54 | –0.16 |
| *MYC* | 0.29 | –2.74 | –0.65 | –1.12 | –1.07 | –0.58 |
| *DTX1* | –0.41 | –1.19 | –0.73 | N.S. | N.S. | N.S. |

Numbers indicate $\log_2$ ratios of mRNA expression in leukemia cells treated with artemisinin (ART), PTC-209 (PTC), PRT4165 (PRT), or *BMI1* siRNA (siRNA) for 24 h normalized to that in DMSO-treated or control siRNA-treated cells. N.S.: Not significantly expressed.

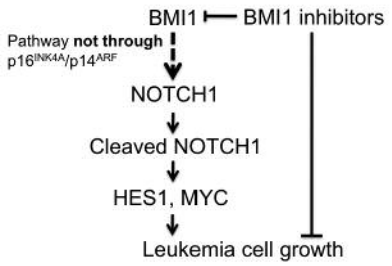


Figure 5. *Schematic representation of the results obtained in this study.*

by BMI1 inhibitors may be used to suppress proliferation of T-ALL cells. However, it is unclear whether BMI1 inhibitors slowed proliferation of leukemic cells through apoptosis related to the down-regulation of NOTCH. In terms of mechanisms of apoptosis, PRT4165 induced caspase-3 cleavage in five cell lines, whereas PTC-209 induced it only in two. More studies should be performed to further understand the molecular mechanisms linking BMI1 inhibition, NOTCH signaling, and growth of leukemic cells.

Considering that BMI inhibitors did not significantly affect the viability of normal lymphocytes, our data indicate that they could be candidates for novel molecular targeting drugs against leukemia. Because BMI1 regulates cell stemness (5, 6), BMI inhibitors could be used as drugs targeting leukemia stem cells. However, more research should be conducted to clarify molecular pathways targeted by BMI inhibitors and ensure their safety for normal hematopoietic stem cells.

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