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Preclinical activity of the novel B-cell-specific Moloney murine leukemia virus integration site 1 inhibitor PTC-209 in acute myeloid leukemia: Implications for leukemia therapy

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Curing patients with acute myeloid leukemia (AML) remains a therapeutic challenge. The polycomb complex protein B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) is required for the self-renewal and maintenance of leukemia stem cells. We investigated the prognostic significance of BMI-1 in AML and the effects of a novel small molecule selective inhibitor of BMI-1, PTC-209. BMI-1 protein expression was determined in 511 newly diagnosed AML patients together with 207 other proteins using reverse-phase protein array technology. Patients with unfavorable cytogenetics according to Southwest Oncology Group criteria had higher levels of BMI-1 compared to those with favorable ($P = 0.0006$) or intermediate cytogenetics ($P = 0.0061$), and patients with higher levels of BMI-1 had worse overall survival (55.3 weeks *vs*. 42.8 weeks, $P = 0.046$). Treatment with PTC-209 reduced protein level of BMI-1 and its downstream target mono-ubiquitinated histone H2A and triggered several molecular events consistent with the induction of apoptosis, this is, loss of mitochondrial membrane potential, caspase-3 cleavage, BAX activation, and phosphatidylserine externalization. PTC-209 induced apoptosis in patient-derived $CD34^+CD38^{low/-}$ AML cells and, less prominently, in $CD34^-$ differentiated AML cells. BMI-1 reduction by PTC-209 directly correlated with apoptosis induction in $CD34^+$ primary AML cells ($r = 0.71$, $P = 0.022$). However, basal BMI-1 expression was not a determinant of AML sensitivity. BMI-1 inhibition, which targets a primitive AML cell population, might offer a novel therapeutic strategy for AML.

Despite intensive chemotherapy and stem cell transplantation for improving the outcome of acute myeloid leukemia (AML), a considerable number of patients with AML eventually relapse and die of the disease. Cancer stem cells were defined originally in AML.[1–3] Leukemia stem cells have been reported to be constitutively resistant to conventional chemotherapy;[4–6] therefore, a novel therapeutic strategy for killing leukemia stem cells is urgently needed. Accumulated data have suggested that B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) is essential for the proliferating and self-renewing ability of normal hematopoietic and leukemia stem cells.[7–9] *BMI-1* has been reported as an oncogene. BMI-1 has been reported to collaborate with BCR-ABL in leukemic transformation.[10] In clinical samples, the mRNA and protein expression levels of BMI-1 have been reported to be higher in $CD34^+$ cells from chronic myeloid leukemia patients compared with those from healthy individuals and, interestingly, BMI-1 expression has been shown to be increased with disease progression from the chronic to advanced phase,[11,12] indicating that BMI-1 confers leukemia stemness and resistance to chemotherapy. In myelodysplastic syndrome, higher protein or transcript levels of BMI-1 have been associated with higher international prognostic scoring system scores or increased blast counts.[13,14] Two studies have suggested that overexpression of BMI-1 may be associated with rapid disease progression and poor outcome for AML patients, although the sample number was relatively small in one study,[15] and BMI-1 expression was determined only at the mRNA level in another.[16] Importantly, knockdown of BMI-1 causes apoptosis and reduces self-renewal in leukemia stem cells.[17] Therefore, the therapeutic targeting of BMI-1, if achieved, would be an attractive strategy for leukemias, particularly for AML, and it remains a therapeutic challenge.

Recently, a selective transcriptional inhibitor of BMI-1, PTC-209, has been developed by a proprietary drug discovery platform technology called GEMS (Gene Expression Modulation by Small-molecules).[18] PTC-209 has been reported to inhibit colorectal cancer-initiating cell self-renewal *in vitro* and effectively block tumor growth in mouse xenografts in the absence of apparent systemic toxicity.[18] In this study, we

investigated the prognostic significance of BMI-1 and the effects of the novel small-molecule selective BMI-1 inhibitor PTC-209 in AML.

Materials and Methods

Reagents. The selective transcriptional BMI-1 inhibitor, PTC-209, was purchased from Xcess Biotechnology (San Diego, CA, USA). The pan-caspase inhibitor Z-VAD-FMK was purchased from Alexis (San Diego, CA, USA).

Cell lines, primary samples, and cell cultures. MOLM-13, MOLM-14, OCI-AML2, OCI-AML3, OCI-AML5, MV4-11, U-937, and HL-60 cells were derived from AML patients, and Reh, NALM-6, MOLT-4, Jurkat, and Raji cells were from acute lymphoblastic leukemia (ALL) patients. MOLM-13, MOLM-14, OCI-AML3, OCI-AML2, OCI-AML5, and NALM6 cells were purchased from DSMZ (Braunschweig, Germany), and the remaining cells were from ATCC (Rockville, MD, USA). Heparinized peripheral blood samples were obtained from AML patients after informed consent according to institutional guidelines per the Declaration of Helsinki. Mononuclear cells were purified by density-gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) separation. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, except for OCI-AML5 that requires MEMα supplemented with 20% FBS and 10% supernatant from the 5637 cell line. Cell viability was evaluated by triplicate counts of Trypan blue dye-excluding cells.

Transfection of BMI-1 siRNA. MOLM-13 cells were transfected with siRNA oligonucleotides by Amaxa Nucleofector 2b, using the cell line Nucleofector kit C (program X-001) according to the manufacturer's instructions (Lonza, Basel, Switzerland). Cells were transfected with negative control siRNA or with BMI-1 siRNA (HSS101040; Life Technologies, Carlsbad, CA, USA). To evaluate the transfection efficiency, cells were transfected with pmaxGFP (Lonza); efficiency of transfection was estimated to be approximately 70%, with approximately 75% cell viability.

Apoptosis analysis. Flow-cytometric determination of annexin V binding, mitochondrial membrane potential loss ($\Delta\Psi_m$), conformational change in BAX, and caspase-3 cleavage were carried out.[19]

Simultaneous detection of intracellular BMI-1, surface CD34, and annexin V by flow cytometry. Cells were stained with annexin V–FITC, fixed with BD Cytofix Fixation buffer and permeabilized with BD Phosflow Perm Buffer III (BD Biosciences, San Jose, CA). Cells were stained with Alexa Fluor 647-conjugated anti-BMI-1 and PE-conjugated anti-CD34 or with isotype controls (BD Bioscience). The BMI-1 expression level was measured as the mean fluorescence intensity ratio (MFIR) calculated by the formula MFIR = (MFI for anti-BMI-1 antibody)/(MFI for isotype control).

Cell cycle analysis. Cell cycle distribution was determined by the Click-It EdU incorporation Kit (Life Technologies). DNA content was determined by propidium iodide staining.

Western blot analysis. Western blot analysis was carried out as previously described.[19] The following antibodies were used: rabbit polyclonal anti-BMI-1 (Proteintech, Chicago, IL, USA), mouse monoclonal anti- β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-ubiquitinated histone H2A (uH2A; Cell Signaling Technology), mouse monoclonal anti-histone H2A (Cell Signaling Technology), and rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology).

Quantitative real-time PCR. The mRNA expression levels were quantified using *Taq*Man gene expression assays (CDKN2A, Hs00923894_m1; CCNG2, Hs00171119_m1; 18S, Hs99999901_s1) (Applied Biosystems, Foster City, CA, USA), as previously described.[19] The level of expression was calculated based on the PCR cycle number (Ct) at which the exponential increase in fluorescence from the probe exceeds a certain threshold value. For each sample, the relative gene expression level was determined by subtracting the Ct value of the housekeeping gene 18S rRNA to the Ct value of the target gene ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{18S rRNA}}$). Relative quantification (fold change) between different samples (e.g., treated *vs*. control) was then determined according to the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{\text{treated sample}} - Ct_{\text{control sample}}$).

Reverse-phase protein array. Peripheral blood and marrow specimens were collected from 511 patients with newly diagnosed AML evaluated at The University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). Normal marrow $CD34^+$ cells from healthy donors were obtained at the same institution. The demographics and clinical characteristics of the patient population have been previously reported.[20,21] The methodology and validation of the proteomic profiling technique have been described previously.[20,22] A validated rabbit polyclonal anti-BMI-1 antibody (Cell Signaling Technology) was used.

Statistical analysis. Statistical analysis was appropriately carried out using the two-tailed Student's *t*-test, Mann–Whitney *U*-test, or paired *t*-test. *P*-values < 0.05 were considered statistically significant. Unless otherwise indicated, average values are expressed as the mean ± SD. Statistical analysis of reverse-phase protein array (RPPA) data was carried out as previously described.[20] Associations between BMI-1 level and categorical clinical variables were assessed using the R software program (version 2.8.0, https://www.r-project.org) with standard *t*-tests, linear regression, or mixed-effects linear models. Associations between protein levels and continuous variables were assessed using Pearson's and Spearman's correlation and linear regression analysis. To correlate the expression of BMI-1 with that of other proteins, we accounted for multiple testing using Bonferroni's correction and accepted any proteins with a Pearson correlation of $|R| \geq 0.2$ and $P < 0.001$. The Kaplan–Meier method was used to generate survival curves, and the log–rank test was used to test the survival difference among the groups of subjects with different protein levels. Patients with missing data were excluded from analysis.

Results

High BMI-1 expression is an adverse prognostic factor in AML. BMI-1 expression levels were determined in bulk AML blasts from 511 newly diagnosed patients using an RPPA, a high-throughput antibody-based technique with procedures similar to that of Western blots. The BMI-1 antibody used for RPPA was validated against five AML cell lines, and the Pearson correlation coefficient between RPPA signals and Western blot intensities was 0.79, which met our criteria for RPPA ($r > 0.7$). BMI-1 expression was significantly higher in the Southwest Oncology Group's unfavorable cytogenetics group ($n = 252$) compared with the intermediate ($n = 225$) or favorable cytogenetics groups ($n = 34$) (median relative levels in the log2 scale: 0.068 *vs*. −0.116, $P = 0.0061$ and 0.068 *vs*.

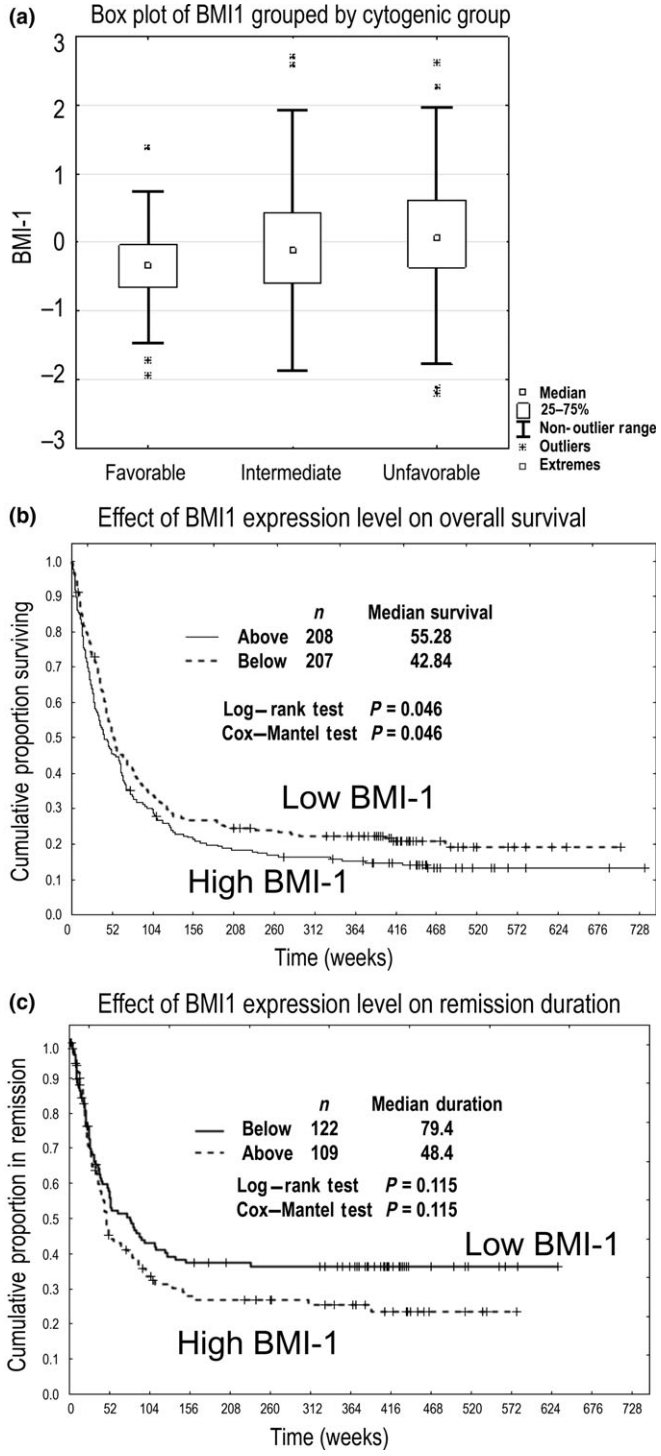

Fig. 1. Increased B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) expression predicts for poor survival in acute myeloid leukemia patients. (a) BMI-1 protein levels in different cytogenetic risk groups that were defined according to the Southwest Oncology Group criteria. (b, c) Kaplan–Meier curves for overall survival (b) and remission duration (c) for patients with acute myeloid leukemia. Patients were dichotomized into two groups at the median level of BMI-1. Patients with higher BMI-1 levels showed worse overall survival.

−0.338, $P = 0.0006$, respectively) (Fig. 1a). Survival information was available for 415 patients. When patients were dichotomized into two groups according to the median level of expression, patients with high levels of BMI-1 had significantly shorter median overall survival (42.8 *vs*. 55.3 weeks, $P = 0.046$) (Fig. 1b). Higher BMI-1 levels did not affect the overall survival of each cytogenetic group (27 *vs*. 37 weeks, $P = 0.26$ for the unfavorable cytogenetics group; 69.7 *vs*. 68 weeks, $P = 0.98$ for the intermediate cytogenetics group; an unachieved, $P = 0.84$ for the favorable cytogenetics group). Remission duration information was available for 231 patients, and patients with higher BMI-1 levels appeared to have inferior relapse-free survival, although the difference was not statistically significant (48.4 *vs*. 79.4 weeks, $P = 0.115$) (Fig. 1c). BMI-1 levels were not significantly correlated with the percentage of $CD34^+$ cells in the AML samples ($r = 0.07$; $P = 0.09$). There was no significant correlation between BMI-1 expression level and white blood cell counts, hemoglobin level, platelet count, or percentages of blasts in the marrow or peripheral blood. The BMI-1 levels in AML samples did not differ from those in normal marrow $CD34^+$ cells ($n = 21$) ($P = 0.773$) (Fig. S1). The mean BMI-1 level in patients with the French–American–British (FAB) M0 subtype was highest among the M0, M1, M2, M3 and M4 subtypes (Fig. S2), raising the possibility that BMI-1 expression is negatively correlated with differentiation. The BMI-1 levels were not significantly affected by the mutational status of Fms-like tyrosine kinase 3 (*FLT3*) or nucleophosmin 1 (*NPM1*). As part of a broader proteomic profiling study of AML, this same RPPA was also probed with antibodies against 207 other proteins.[23] Of these, 77 proteins were found to be positively ($n = 45$) or negatively ($n = 32$) correlated with the BMI-1 level ($|R| \geq 0.2$, $P < 0.001$) (Fig. S3). BMI-1 levels were positively correlated with exportin 1 (XPO1), forkhead homeobox type O 3A (FOXO3A), Musashi RNA binding protein 2 (MSI2), and cAMP-responsive element binding protein 1 (CREB1), and they were negatively correlated with phosphatase and tensin homolog deleted on chromosome 10 (PTEN).

PTC-209 mediates growth arrest and apoptosis in AML. We examined the effects of PTC-209 on the cellular viability of eight AML and five ALL cell lines. The ED_{50} and IC_{50} values at 48 h are summarized in Table 1. PTC-induced cell death, as evidenced by the percentage of Trypan blue uptake, was directly correlated with annexin V induction in leukemia cells (data not shown). PTC-209 induced a dose-dependent annexin V induction in the AML and ALL cell lines (Fig. 2a,b). PTC-209 also showed a time-dependent annexin V induction in sensitive AML cell lines beginning at 12 h (Fig. 2c). There were three PTC-resistant ($ED_{50} > 10$ μM) cell lines, and they were all derived from ALL (Table 1). All eight AML cell lines had an $ED_{50} < 3$ μM, whereas only two out of the five ALL cells had an $ED_{50} < 3$ μM. These data raise the possibility that AML cells are more sensitive to PTC-209-induced apoptosis than ALL cells. In contrast, PTC-209 showed similar antiproliferative effects to the AML and ALL cell lines, and the IC_{50} levels were not significantly different between the AML and ALL cell lines ($P = 0.19$) (Table 1).

Next, we investigated whether there was a relationship between the basal level of BMI-1 and cell sensitivity to PTC-209. We quantified the BMI-1 levels in cell lines using MFIR, and they were normalized to that of OCI-AML3 cells (Table 1). The basal level of BMI-1 was not correlated with the ED_{50} ($r = -0.42$, $P = 0.15$) (Fig. 3a) or IC_{50} ($r = -0.32$, $P = 0.29$) (Fig. 3b), implying that basal BMI-1 levels may not be a determinant of PTC-209 sensitivity.

PTC-209 reduces levels of BMI-1 and uH2A followed by mitochondrial apoptosis in AML cells. The impact of PTC-209 on the protein levels of BMI-1 and its downstream target uH2A

Table 1. ED_{50}, IC_{50} and BMI-1 protein levels in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) cell lines

	Cell lines	ED_{50} (μM)	IC_{50} (μM)	BMI-1 levels (MFIR)
AML cell lines	MOLM-13	1.5	0.44	3.7
	OCI-AML3	2.6	0.51	1.0
	MV4-11	1.2	0.26	1.1
	OCI-AML5	2.7	0.70	1.4
	HL-60	2.5	0.23	0.7
	U-937	1.6	0.21	5.3
	MOLM-14	2.6	0.22	4.7
	OCI-AML2	1.8	0.77	2.6
ALL cell lines	Reh	>10.0	0.89	1.6
	NALM6	>10.0	0.47	1.4
	Jurkat	2.6	0.53	3.9
	Raji	>10.0	0.52	0.8
	MOLT-4	1.4	0.32	5.8

MFIR, mean fluorescence intensity ratio.

was determined. In MOLM-13 cells, PTC-209 decreased the BMI-1 and uH2A levels by 63% and 57%, respectively, at 24 h of treatment (Fig. 4a). Decreases in the BMI-1 and uH2A levels were observed as early as 2 h after exposure to 5 μM PTC-209. The cleavage of caspase-3 was evident at 16 h, indicating that inhibition of BMI-1 and consequent uH2A reduction occurs prior to activation of the apoptosis machinery. PTC-209 induced caspase-3 cleavage (Fig. 4b), BAX conformational change (Fig. 4c), and $\Delta\Psi_m$ loss (Fig. 4d), indicating mitochondrial apoptosis. Similar results were obtained in MV4-11 cells (data not shown). It has been reported that BMI-1 knockdown induces apoptosis in normal hematopoietic and leukemic cells.[17] To further investigate the role of BMI-1 in AML cell survival, BMI-1 levels were reduced in MOLM-13 cells using siRNA. BMI-1 siRNA led to an inhibition of BMI-1 expression relative to cells transfected with a control siRNA 24 h after transfection (Fig. S4). We did not detect interference with the synthesis of the housekeeping cellular protein β-actin. BMI-1 knockdown reduced the uH2A level at 24 h and induced PARP cleavage at 48 h after transfection (Fig. S4).

Because PTC-209 showed high antiproliferative effects on all cell lines examined, we investigated the cell cycle distribution of two AML cell lines (MOLM-13 and OCI-AML3) that were treated with PTC-209 at 2 × IC_{50}. At this concentration, PTC-209 did not induce apoptosis. After 48 h, cells accumulated in G_1 phase, while the percentage of S phase cells decreased, indicating that PTC-209 impairs S phase entry. For MOLM-13 cells, PTC treatment led to a higher percentage of cells in G_1 phase (68 ± 0.4% compared with 60 ± 0.6% for untreated cells, $P = 0.0008$) and a lower percentage of cells in S phase (20 ± 0.5% *vs.* 33 ± 0.1%, $P = 0.0047$) (Fig. S5). Similar results were obtained for OCI-AML3 cells (data not shown). BMI-1 has been shown to repress the *CDKN2A* gene (encoding INK4a/ARF).[24] INK4a inhibits the cyclin-dependent kinases CDK4 and CDK6, which are critical for G_1 progression and G_1/S transition. It is therefore anticipated that PTC-209 may cause G_1 arrest through CDKN2A induction. However, a recent study has shown that BMI-1 inhibition by PTC-209 induces the *CCNG2* gene (encoding cyclin G2).[25] Cyclin G2 inhibits CDK2 activity and causes G_1/S arrest.[26] To investigate if PTC-209 treatment induces *CDKN2A* and *CCNG2*, MOLM-13 cells were treated for 24 h with PTC-209 at 2 × IC_{50}, and *CDKN2A* and *CCNG2* transcripts were quantitated by real-time PCR. PTC-209 treatment led to a significant induction of both *CDKN2A* and *CCNG2* (Fig. S6).

PTC-209 induces apoptosis in $CD34^+CD38^{low/-}$ primitive leukemia cells from AML patients. We examined the apoptotic effects of PTC-209 on primary cells from 22 patients with AML. The patient characteristics are summarized in Table 2. Twelve patients had newly diagnosed AML, and the remaining 10 patients had relapsed or refractory disease. Cytogenetically, 10 cases had an intermediate karyotype, and 12 had an unfavorable karyotype (7 with a complex karyotype and 5 with another unfavorable karyotype) according to the Southwest Oncology Group's cytogenetic risk criteria. The *FLT3* and *NPM1* mutational status was available for all samples. Treatment of the 22 primary AML samples with PTC-209 resulted in a dose-dependent increase in the percentage of annexin V-positive cells (Fig. 5a). The percentage of cells undergoing PTC-209-mediated apoptosis at 48 h after exposure to 0.5, 1, and 2 μM PTC-209 was 3.1 ± 1.0%, 22.9 ± 4.8%, and 43.8 ± 5.6% (mean ± SEM), respectively. The cell susceptibility varied widely among samples. Importantly, the percentages were significantly higher for $CD34^+CD38^{low/-}$ cells

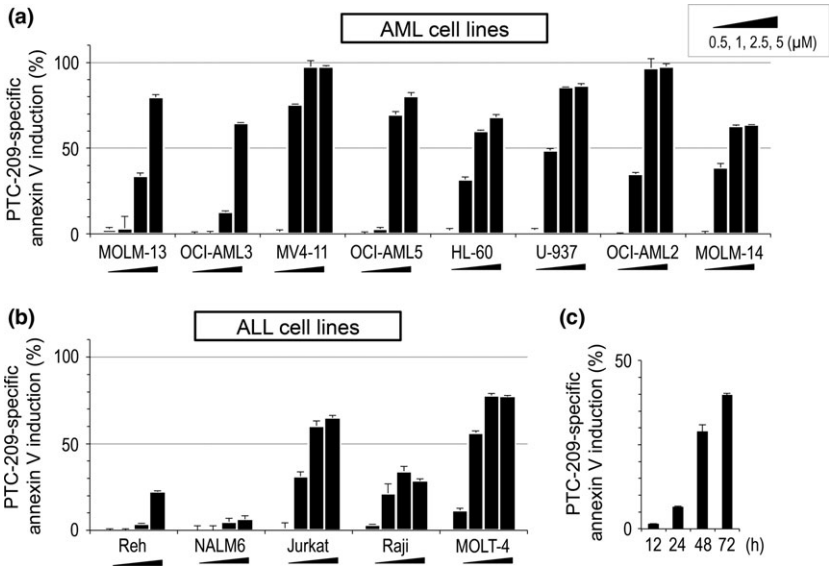

Fig. 2. B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) inhibitor PTC-209 induces apoptosis in leukemia cells. (a,b) Acute myeloid leukemia (AML) (a) and acute lymphoblastic leukemia (ALL) (b) cells were incubated with 0.5, 1, 2, or 5 μM PTC-209 for 48 h, and annexin V-positive fractions were measured by flow cytometry. (c) MOLM-13 cells were treated with 2.5 μM PTC-209 for the indicated times, and annexin V-positive fractions were measured. The results are expressed as the mean ± SD of triplicate measurements.

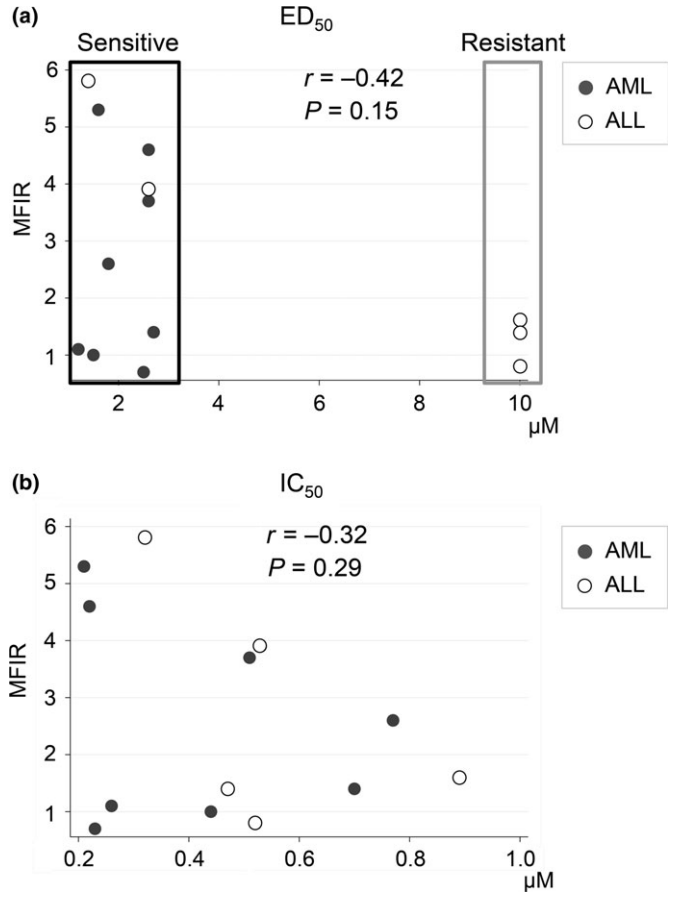

Fig. 3. Basal B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) expression does not determine PTC-209 sensitivity in acute myeloid leukemia (AML). There was no significant correlation between the basal BMI-1 level and the ED_{50} (a) or IC_{50} (b) values. BMI-1 levels were quantified in cell lines using mean fluorescence intensity ratio (MFIR), normalized to that of OCI-AML3 cells, and correlated with the ED_{50} or IC_{50}. ALL, acute lymphoblastic leukemia.

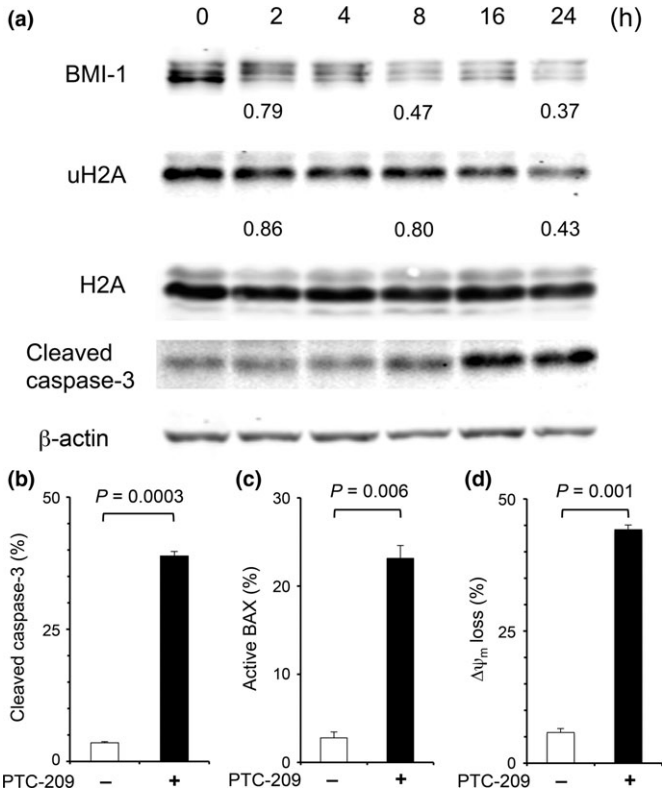

Fig. 4. PTC-209 reduces the B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) protein level and induces mitochondrial apoptosis in acute myeloid leukemia. (a) Expression of BMI-1 and ubiquitinated histone H2A (uH2A) in MOLM-13 cells, which were treated with 5 μM PTC-209 for the indicated times. The intensity of the immunoblot signals was quantified, and the relative intensities compared with β-actin for BMI-1 and total H2A for uH2A were calculated. The results are representative of three independent experiments. (b) Caspase-3 cleavage was determined by flow cytometry in MOLM-13 cells after an 18-h exposure to 5 μM PTC-209. (c) BAX conformational changes were determined by using conformation-specific anti-BAX antibody YTH-6A7 in MOLM-13 cells treated with 5 μM PTC-209 for 18 h. The pan-caspase inhibitor Z-VAD-FMK (50 μM) was used to inhibit caspase-induced conformational changes in BAX. (d) MOLM-13 cells were treated with 5 μM PTC-209 for 24 h, and $\Delta\Psi_m$ loss was determined. The results are expressed as the mean ± SD. Comparable results were obtained in two additional independent experiments.

compared with $CD34^-$ AML cells (33.4 ± 6.2% *vs.* 15.9 ± 4.2%, $P = 0.0001$ at 1 μM PTC-209 and 57.1 ± 6.5% *vs.* 27.5 ± 5.2%, $P < 0.0001$ at 2 μM PTC-209) (Fig. 5a), indicating that primitive leukemia cells are more susceptible to BMI-1 inhibition-induced apoptosis than mature AML cells. The percentage of blasts or $CD34^+$ cells did not correlate with 1 μM PTC-209-specific annexin V induction ($r = 0.07$, $P = 0.77$ and $r = -0.07$, $P = 0.76$, respectively). The AML cell sensitivity was independent of disease status, cytogenetic criteria, and *FLT3* or *NPM1* mutation status. The percentage of specific apoptosis induced by 1 μM PTC-209 was 21.9 ± 5.5% for *FLT3* wild-type samples and 24.2 ± 9.2% for *FLT3* mutant samples ($P = 0.87$), and it was 22.7 ± 5.3% for *NPM1* wild-type samples and 24.5 ± 7.5% for *NPM1* mutant samples ($P = 0.57$). We correlated baseline BMI-1 protein levels as determined by Western blotting with the extent of PTC-induced annexin V in 10 samples with AML. However, there was no significant correlation in bulk cells ($r = 0.36$, $P = 0.303$ at 1 μM PTC-209; $r = 0.35$, $P = 0.318$ at 2 μM PTC-209) and $CD34^+CD38^{low/-}$ immature cells ($r = 0.52$, $P = 0.12$ at 1 μM PTC-209; $r = -0.11$, $P = 0.761$ at 2 μM PTC-209).

BMI-1 has been shown to be important for the maintenance of normal hematopoietic stem cells and AML leukemia stem cells. We investigated the apoptotic effects of PTC-209 on normal hematopoietic cells. First, we exposed circulating normal lymphocytes to PTC-209. As shown in Figure 5(b), normal lymphocyte samples were resistant to PTC-induced apoptosis. Even at the highest concentration of PTC-209 (10 μM), normal lymphocytes underwent minimal apoptosis (10.4 ± 4.6% PTC-induced annexin V-positive cells). Next, the sensitivity of normal bone marrow cells to PTC-induced apoptosis was determined. PTC-209 induced modest annexin V induction in bulk bone marrow mononuclear cells (7.1 ± 1.5% and 13.0 ± 4.3% PTC-specific annexin V induction at 1 and 2 μM, respectively). In contrast, $CD34^+CD38^{low/-}$ immature hematopoietic cells were susceptible to PTC-209 (37.9 ± 6.5% and 62.0 ± 3.1% PTC-specific annexin V induction at 1 and 2 μM, respectively) (Fig. 5c).

PTC-209-induced BMI-1 reduction is associated with apoptosis induction in $CD34^+$ primary AML cells. To investigate the relationship between the reduction in BMI-1 protein levels and apoptosis induction by PTC-209 in primary AML samples, we established a triple staining assay for BMI-1, CD34, and

Table 2. Clinical data for patients with acute myeloid leukemia

No.	Age, years/sex	Status	Cytogenetics	Source	Blasts, %	CD34, %†
1	69/M	Relapse	Complex	PB	84	1.4
2	69/M	Relapse	Complex	BM	74	1.3
3	25/M	Relapse	46,XY,t(8;21)(q22;q22),del(9)(q22;q34)	PB	84	47.9
4	68/F	New	46,XX	PB	61	26.4
5	66/M	New	48,XY,+13,+15	BM	42	12.5
6	76/M	New	46,XY	PB	66	18.7
7	65/M	Relapse	Complex	BM	93	3.0
8	29/F	New	Complex	PB	97	59.8
9	71/M	New	46,XY	PB	23	14.3
10	57/M	Refractory	Complex	PB	87	17.1
11	81/F	New	Complex	PB	86	0.8
12	71/F	New	Complex	PB	57	8.0
13	24/M	Relapse	47,XY,t(6;9)(p23;q34),+8	PB	97	21.1
14	33/M	Relapse	46,XY,t(3;7)(p21;q36)	PB	84	0.9
15	59/F	Refractory	46,XY,del(5)(q22;q35)	PB	48	4.9
16	30/M	New	46,XY	PB	38	10.6
17	62/M	New	46,XY,del(7)(q32)	PB	43	6.3
18	62/M	Refractory	46,XY,del(13)(q12q14)	PB	95	9.6
19	73/F	New	46,XX	PB	81	6.6
20	58/M	Relapse	45,XY,-7	PB	52	2.3
21	70/M	New	46,XY	BM	21	2.5
22	65/F	New	46, XX, t(6;11)(q27;q23)	PB	21	2.5

†CD34 positivity was determined by flow cytometry in bone marrow (BM) or peripheral blood (PB) mononuclear cells. F, female; M, male.

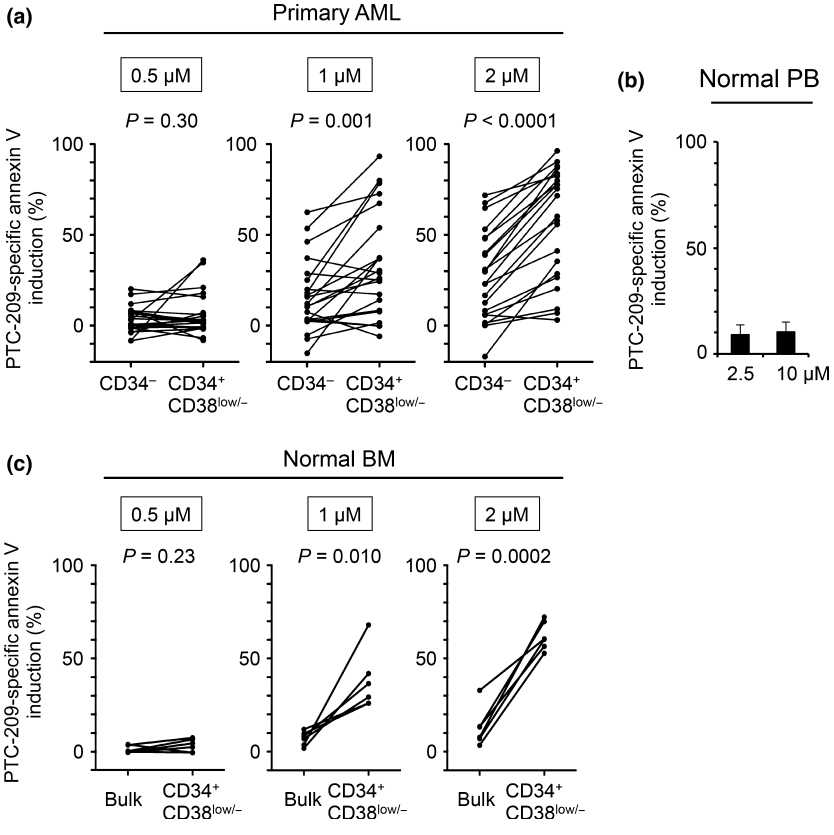

Fig. 5. PTC-209 induces apoptosis in primary acute myeloid leukemia (AML) samples, particularly in the $CD34^+CD38^{low/-}$ cell population. Twenty-two primary AML samples (a), three normal peripheral blood (PB) lymphocyte (b), and six normal bone marrow (BM) cells (c) were incubated with the indicated concentrations of PTC-209 for 48 h, and annexin V-positive fractions were measured by flow cytometry.

annexin V. A total of 10 AML samples were studied. Basal levels of BMI-1 expression expressed as MFI relative to isotype control were 3.4 ± 0.4 times higher in $CD34^+$ compared to $CD34^-$ cells ($P = 0.0002$). PTC-209 treatment resulted in decreased levels of BMI-1 in $CD34^+$ and $CD34^-$ cells. The reduction in BMI-1 protein level with 1 μM PTC-209 was significantly higher in $CD34^+$ compared with $CD34^-$ cells (35.8 ± 5.5% *vs.* 13.9 ± 2.8%, $P = 0.013$) (Fig. 6a). The proportion of PTC-specific annexin V-positive cells was significantly higher in $CD34^+$ than $CD34^-$ cells (21.4 ± 7.3% *vs.*

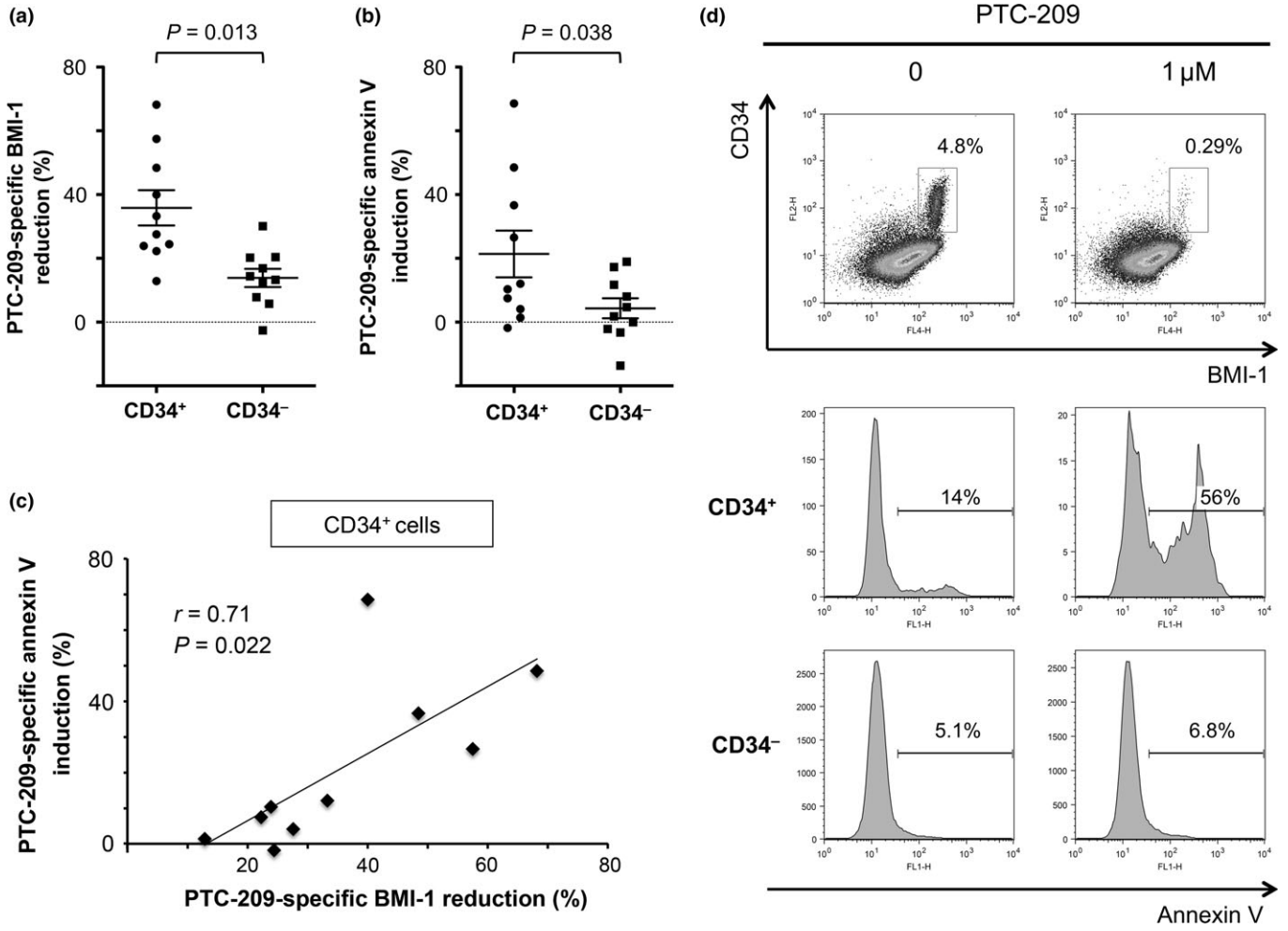

Fig. 6. PTC-209 eradicates $CD34^+$ acute myeloid leukemia (AML) cells together with reduced B-cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) expression. (a) Correlation between the extent of BMI-1 reduction and degree of apoptosis induced by PTC-209 in $CD34^+$ AML cells. (b) A primary AML sample was treated with 1 μM PTC-209 for 48 h. $BMI\text{-}1^{high}CD34^+$ cells were gated in dot plots (upper row). Histograms indicate the distribution in annexin V binding in $CD34^+$ (middle row) and $CD34^-$ (lower row) AML cell fractions.

4.3 ± 3.2%, $P = 0.038$) (Fig. 6b). In the $CD34^+$ fraction, the reduction in BMI-1 was positively correlated with the percentage of annexin V-induced cells ($r = 0.71$, $P = 0.022$) (Fig. 6c), while the basal BMI-1 levels were not correlated with the percentage of annexin V-induced cells ($r = -0.45$, $P = 0.19$), indicating that BMI-1 reduction may predict apoptosis induction in $CD34^+$ cells. In the $CD34^-$ population, the reduction in BMI-1 was not correlated with the percentage of annexin V-induced cells ($r = 0.48$, $P = 0.16$). Figure 6(d) shows a representative example of a distinct cell population in which $CD34^+BMI\text{-}1^{High}$ expression is eradicated by PTC-209 treatment (upper row).

Discussion

We profiled the BMI-1 protein level in a large cohort of 511 AML patients using RPPA and found that BMI-1 protein levels are prognostic for AML. The BMI-1 protein level was significantly higher in patients with unfavorable cytogenetics compared with those with intermediate or favorable cytogenetics. Inferior overall survival for the high BMI-1 expression group provides an additional prognostic definition and rationale for therapeutically targeting BMI-1. The BMI-1 levels were not directly correlated with *FLT3* or *NPM1* mutations. Considering the limited therapeutic choices for refractory or relapsed AML, our findings support the idea that targeting BMI-1 is an attractive therapeutic approach for AML, particularly for AML cases with an unfavorable prognosis.

The regulation of the cellular expression of BMI-1 in AML has been poorly understood. Using RPPA, we found a positive correlation between BMI-1 protein levels and the stem cell maintenance proteins FOXO3, MSI2 and CREB1.[17,27,28] FOXO3 is overexpressed in relapsed AML samples,[29] and the accumulation of nuclear FOXO3 is associated with drug resistance.[30] MSI2 and CREB1 are also overexpressed in AML and negatively impact survival.[31,32] In addition, XPO1 was one of the most positively correlated proteins with BMI-1. Previously, we identified XPO1 as an independent prognostic factor for AML,[23] and more recently, XPO1 has been reported to be associated with BCR/ABL-independent resistance to tyrosine kinase inhibitors in chronic myeloid leukemia.[33] In contrast, BMI-1 demonstrated a negative correlation with PTEN. PTEN has been found to bind BMI-1 and inhibit its function,[34] and BMI-1 has been reported to suppress PTEN.[35] These results improve our understanding of BMI-1 regulation in AML and could guide strategies for targeting this population.

We found that BMI-1 inhibition by PTC-209 induces mitochondrial apoptosis in AML cells. In accordance with a previous study,[18] PTC-209 decreased the BMI-1 level and the downstream target of PRC1 uH2A. Interestingly, the baseline levels of BMI-1 were not associated with the extent of PTC-induced apoptosis, indicating that the abundance of the target protein is not a determinant for PTC-induced apoptosis. In contrast, there was a positive correlation between the extent of the reduction of BMI-1 levels and the PTC-induced apoptosis level in $CD34^+$ AML cells. Our data indicate that treatment-related BMI-1 reduction may be a response biomarker for BMI-1 inhibition in AML. The PTC-induced reduction in BMI-1 was more prominent in $CD34^+$ AML cells compared with $CD34^-$ AML cells, and in accordance with these data, the $CD34^+CD38^{low/-}$ primitive AML population was highly susceptible to PTC-209. PTC-209 rapidly (as early as 2 h) reduced the BMI-1 cellular levels, arguing against the idea that reduced BMI-1 levels occur as a result of cell death in PTC-treated cells. Recently, a more potent, orally active BMI-1 inhibitor, PTC596, has been developed.[36] Taken together, BMI-1 inhibition may be a novel therapeutic strategy to kill leukemia stem cells.

One concern is that BMI-1 inhibition might affect normal hematopoietic systems and leukemia proliferation because BMI-1 is important for the maintenance of normal hematopoietic stem cells and leukemia stem cells. Although BMI-1 inhibition by PTC-209 has been shown to be minimally toxic for normal hematopoiesis in mouse models,[18] our data demonstrated that PTC-209 might affect normal hematopoietic stem cells. The hypothesis that BMI-1 inhibition has an optimal therapeutic index for hematologic toxicities is now validated in patients undergoing clinical trials with the clinical BMI-1 inhibitor PTC596 (NCT02404480).

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. BMI-1 expression in acute myeloid leukemia blasts and normal $CD34^+$ bone marrow cells.

Fig. S2. BMI-1 expression in different FAB subtypes.

Fig. S3. List of proteins significantly correlated with BMI-1 expression.

Fig. S4. BMI-1 knockdown induces PARP cleavage.

Fig. S5. PTC-209 blocks G_1/S transition.

Fig. S6. Expression levels of *CDKN2A* and *CCNG2* after 24 h of treatment with 0.9 μM (= 2 × IC_{50}) PTC-209 in MOLM-13 cells.