Panobinostat inhibits the proliferation of CD34^{+}CD38^{-}cells under stimulation of hematopoietic growth factors on AGM-S3 cells in juvenile myelomonocytic leukemia

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Abbreviations	
JMML	Juvenile myelomonocytic leukemia
AGM	aorta-gonad-mesonephros
SCF	stem cell factor
TPO	thrombopoietin
CB	cord blood

Abstract

Background

Encouraging responses to Histon deacetylase (HDAC) inhibitors have been reported for hematologic malignancies. Here we report effects of panobinostat and 5-azacytidine on the proliferation of juvenile myelomonocytic leukemia (JMML) $CD34^+$ cells.

Procedure

We previously reported that stimulation of JMML $CD34^+$ cells with stem cell factor and thrombopoietin on irradiated murine AGM-S3 cells led to substantial expansion of JMML $CD34^+$ cells that contained leukemic stem cells capable of transplantation into immunodeficient mice. Using this culture system, we evaluated effects of panobinostat and 5-azacytidine on the proliferation of JMML $CD34^+$ cells.

Results

Panobinostat dose dependently reduced the numbers of day 7-$CD34^+$ cells generated under stimulation of hematopoietic growth factors on AGM-S3 cells in all eight patients with JMML. These patients possessed various genetic and/or karyotypic abnormalities. $CD34^+CD38^-$ cells were substantially more sensitive to panobinostat at 10 and 20 nM

than $CD34^+CD38^+$ cells. Panobinostat, however, failed to influence the ability of AGM-S3 cells to stimulate JMML $CD34^+$ cell production. In contrast to HL60 cells, apoptosis and cell cycle arrest in panobinostat-mediated inhibition were at low levels in JMML. The inhibitor also suppressed the factors-dependent proliferation of normal $CD34^+$ cells on AGM-S3 cells. Meanwhile, no substantial inhibitory effects of 5-azacytidine on the growth of JMML $CD34^+$ cells were observed.

Conclusions

These results demonstrate that panobinostat directly suppresses the growth of JMML $CD34^+$ cells, in particular $CD34^+CD38^-$ cells, regardless of the genetic abnormality type, suggesting that it is a useful anti-leukemic drug to target JMML stem cells at a pre-transplant stage.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a fatal, mixed myeloproliferative and myelodysplastic disorder that occurs during infancy and early childhood. Approximately 85% of patients with JMML have genetic abnormalities in the RAS

pathway, such as inactivation of *NF1* or mutations in *PTPN11*, *NRAS*, *KRAS*, and *CBL* [1]. Based on whole-exome sequencing, Sakaguchi *et al* demonstrated that *SETBP1* and *JAK3* mutations are common, recurrent secondary events associated with poor clinical outcomes in this disorder [2]. Our genetic analysis of individual granulocyte-macrophage colonies revealed that these non-RAS pathway gene mutations may represent the second genetic aberration in a proportion of children affected by JMML with *PTPN 11* mutations [3]. Using droplet digital polymerase chain reaction (PCR), Stieglitz *et al* detected *SETBP1* mutations in approximately 30% of patients with JMML, suggesting that subclonal mutations at diagnosis confer a dismal prognosis in JMML [4]. More recently, Caye *et al* reported multiple concomitant genetic hits targeting the RAS pathway and new pathway activation involving phosphoinositide 3-kinase and mTORC2 complex through RAC2 mutation [5]. In addition, their study defined PRC2 loss that switches the methylation/acetylation status of histone H3 lysine 27.

We previously reported that the percentage of patients with JMML who had *p15* hypermethylation was lower than 20% [6]. Subsequent studies elucidated frequent

hypermethylation of significant numbers of genes in this disorder. Olk-Batz *et al* identified that CpG islands of *BMP4*, *CALCA*, *CDKN2B*, and *RARB* genes were frequently hypermethylated in 127 JMML cases [7]. They found that a high methylation phenotype characterizes an aggressive biologic variant of JMML and is an important molecular predictor of the outcome. Epigenetic silencing also occurs in *a-kinase anchor protein 12 (AKAP12)* gene, whose product functions as a regulator of protein kinase A and C signaling, acting downstream of RAS [8]. Cseh *et al* reported that complete clinical, cytogenetic, and/or molecular genetic remissions before allogeneic hematopoietic stem cell transplantation were achieved by treatment with low-dose azacytidine (a DNA methyltransferase inhibitor) in three of 12 patients with JMML [9].

Histone deacetylase (HDAC) inhibitors are a class of antineoplastic agent targeting the epigenome, specifically chromatin remodeling, resulting in modulation of genes responsible for apoptosis and cell cycle regulation, and also hyperacetylation of many non-histone proteins. George *et al* reported that panobinostat induces acetylation of histone H3 and H4 and of heat shock protein 90, increases p21 levels, as well as induces cell-cycle G1 phase accumulation and apoptosis of human myeloid leukemia cell lines

(K562 cells and MV4-11 cells with the activating length mutation of *FLT-3*) [10]. Encouraging responses to HDAC inhibitors including panobinostat have been reported in some clinical trials for hematologic malignancies [11]. However, there have been no clinical trials of HDAC inhibitors for children with JMML.

Maiso *et al* reported that to improve the potency of HDAC inhibitor, the agents that act on cells at nanomolar concentrations are more effective than those act on cells at micromolar concentrations [12]. Additionally, safety to pediatric patients is important for clinical use of an anticancer drug [13]. Accordingly, we selected panobinostat as an HDAC inhibitor that met the two criteria. We previously reported that stimulation of JMML $CD34^+$ cells with stem cell factor (SCF) and thrombopoietin (TPO) during culture on AGM-S3 cells led to substantial expansion of JMML $CD34^+$ cells that contained leukemic stem cells capable of transplantation into immunodeficient mice after 7 days culture [14]. Using this culture system, we evaluated effects of panobinostat on the proliferation of JMML $CD34^+$ peripheral blood (PB) cells, and compared with effects of 5-azacytidine.

Materials and Methods

This study was approved by the institutional review board of Shinshu University School of Medicine. Informed consent was obtained from the guardians of the patients following institutional guidelines.

Cell preparation

JMML $CD34^+$ cells were enriched from PB mononuclear cells (containing 1.3% to 1.5% of $CD34^+$ cells) frozen at the time of disease onset from eight patients by positive immunomagnetic selection using a CD34 MicroBead kit (Miltenyi Biotec, Inc., Auburn, CA, USA). Their genetic and chromosomal abnormalities are shown in Table 1. Flow cytometric analysis revealed that 99% of isolated cells were positive for CD34. Cord blood (CB) $CD34^+$ cells and bone marrow (BM) $CD34^+$ cells were purchased from Riken BioResource Center (Tsukuba, Japan) and STEMCELL Technologies, Inc. (Vancouver, Canada), respectively.

Reagents

Panobinostat (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 5-azacytidine (Sigma Chemical, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide, and then

diluted with alpha-medium.

Suspension cultures

JMML $CD34^+$ cells ($0.4–2 \times 10^4$) were transferred in 35-mm gelatin-coated dishes that had been covered with irradiated confluent AGM-S3 cells. As reported previously [14], the cells were cultured in 10 ng/mL SCF and 10 ng/mL TPO with or without panobinostat at concentrations of 10–40 nM. 5-azacytidine was added at concentrations of 2–8 μM. The plates were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. On day 7, the number of viable cells was determined by a trypan-blue exclusion test. When the cultures were performed up to day 14 or 28, half of the culture medium was replaced every 3 days with fresh medium containing the factors.

Effects of panobinostat or 5-azacytidine on stromal cell function

To examine effects of panobinostat on the stimulatory ability of AGM-S3 cells to generate JMML $CD34^+$ cells, irradiated confluent AGM-S3 cells were cultured with or without 40 nM of panobinostat for 7 days, and then JMML $CD34^+$ cells were seeded with SCF+TPO on the stromal cells rinsed off the reagent with the culture medium. 5-azacytidine was used at a concentration of 8 μM.

Flow cytometric analysis

The analysis of surface expression of CD14, CD15, and CD38 on the progenies generated from JMML $CD34^+$ cells was performed, as described previously [14].

To examine apoptosis and cell cycle, we performed flow cytometric analysis after annexin V/propidium iodide (PI) double staining (Annexin A5-FITC Kit –Apoptosis Detection Kit, Beckman Coulter; Brea, CA, USA), and after staining with 5'-bromo-2'-deoxyuridine (BrdU) and 7-amino-actinomycin D (7-AAD; BrdU Flow Kit, BD Pharmingen™, Santiago, CA, USA).

Fluorescence *in situ* hybridization (FISH) analysis

FISH analysis was performed, using probes specific for the centromere of chromosome 7 and for the centromere of chromosome 8 according to a procedure described previously [15].

Statistical analysis

Values are expressed as means ± SD. To determine the significance of differences, we used one-way analysis of variance, followed by *post hoc* contrasts with Bonferroni limitation. The sensitivity to the reagent between JMML $CD34^+CD38^-$ cells and

$CD34^+CD38^+$ cells was analyzed using the paired *t*-test. The chi-squared test was used to examine effects of the reagents on the growth of progenies with abnormal karyotype. All statistical analyses were performed with EZR [16], and statistical significance was defined as $p < 0.05$.

Results

Effects of panobinostat or 5-azacytidine on the growth of HL-60 and THP-1 cells

We first examined effects of panobinostat or 5-azacytidine on the growth of HL-60 and THP-1 cells. As presented in Supplementary Fig. S1A, panobinostat at concentration of 10 nM or higher significantly suppressed the proliferation of HL-60 and THP-1 cells with estimated IC_{50} values of 13.9 nM and 6.8 nM, respectively. 5-azacytidine also exerted substantial inhibition on the both myeloid leukemic cells: IC_{50} value was 1.2 μM for HL-60 cells and 2.8 μM for THP-1 cells (Supplementary Fig. S1B).

Effects of panobinostat or 5-azacytidine on the proliferation of JMML $CD34^+$, $CD34^+CD38^-$ and $CD34^+CD38^+$ PB cells

Recently, we reported that JMML $CD34^+$ cells grown on irradiated AGM-S3 cells in the presence of SCF+TPO for 7 days contain leukemic stem cells capable of transplantation into immunodeficient mice [14]. PB $CD34^+$ cells isolated from eight patients with JMML (shown in Table 1) expanded to approximately 9.1 ± 7.8 (1.7–22.6)-fold the initial number after 7 days of culture. As presented in Figs. 1A and 1B, the addition of panobinostat (10 nM and 20 nM) profoundly reduced numbers of day 7-$CD34^+$ cells. Nearly complete inhibition was obtained by panobinostat at 40 nM. Interestingly, $CD34^+CD38^-$ cells were substantially more sensitive to 10 nM or 20 nM of panobinostat than $CD34^+CD38^+$ cells. The ability of 20 nM of panobinostat to suppress the growth of JMML $CD34^+CD38^-$ cells was equivalent to that of 40 nM. Estimated IC_{50} values of the inhibitor were 9.2 ± 3.0 nM for JMML $CD34^+$ cells, 6.3 ± 1.0 nM for $CD34^+CD38^-$ cells, and 11.9 ± 4.1 nM for $CD34^+CD38^+$ cells.

However, 5-azacytidine at concentration of 2 μM showed no significant inhibition on the production of JMML $CD34^+$ cells, $CD34^+CD38^-$ cells and $CD34^+CD38^+$ cells under stimulation with SCF+TPO on AGM-S3 cells for 7 days (Fig. 1). Similar results were obtained at concentrations of 4 μM or higher except for $CD34^+CD38^+$ cells in 8 μM.

There was no significant difference in susceptibility to 2 to 8 μM of 5-azacytidine between $CD34^+CD38^-$ cell subset and $CD34^+CD38^+$ cell subset. A possible explanation is degradation of 5-azacytidine in culture. When 4 μM of 5-azacytidine was added every three days up to day 14, the reagent failed to influence the proliferation of JMML $CD34^+$ cells. In addition, culture supernatant obtained 7 days after incubation of HL60 cells with 4 or 8 μM of 5-azacytidine inhibited the growth of HL60 cells to a similar extent to the value achieved by the addition of fresh 5-azacytidine (4 or 8 μM), as shown in Supplementary Fig. S2. The addition of 4 μM of 5-azacytidine failed to augment 10 or 20 nM of panobinostat-induced suppression of JMML $CD34^+$ cell expansion.

We then evaluated effects of panobinostat and 5-azacytidine on mature population ($CD34^-$ cells) generated from JMML $CD34^+$ cells, based on our previous results [14]. As shown in Supplementary Fig. S3, exposure to panobinostat at 10 nM, significantly suppressed the growth of mature population. On the contrary, 5-azacytidine exerted no profound inhibition.

Effects of panobinostat on the stimulatory ability of AGM-S3 cells to generate

JMML $CD34^+$ cells

To elucidate a possibility that panobinostat decreased proliferation of JMML $CD34^+$ cells via stromal cell dysfunction, AGM-S3 cells were incubated with or without 50 nM of the reagent. After 7 days of culture, the viability of AGM-S3 cells was higher than 80% in the both conditions. We then examined whether panobinostat influenced the stimulatory ability of AGM-S3 cells to generate JMML $CD34^+$ cells. Irradiated AGM-S3 cells were cultured with or without 40 nM of panobinostat for 7 days, and then JMML $CD34^+$ cells were seeded with SCF+TPO on the stromal cells rinsed off the reagent with the culture medium. As presented in Fig. 2, there was no significant difference in numbers of day 7-cultured $CD34^+$ cells, $CD34^+CD38^-$ cells, and $CD34^+CD38^+$ cells in the presence or absence of panobinostat. The results were confirmed in the other experiment.

Effects of panobinostat on apoptosis, cell cycle and differentiation of progenies generated from $CD34^+$ cells on AGM-S3 cells in the presence of SCF+TPO

We then evaluated influence of panobinostat on apoptosis, cell cycle and differentiation of progenies generated from JMML $CD34^+$ cells, using flow cytometry

after annexin V/PI staining. As shown in Fig. 3A, a large part of HL60 cells treated with 20 nM of panobinostat were positive for annexin V and/or PI. On the contrary, a frequency of apoptotic population of progenies derived from JMML $CD34^+$ cells after treatment with panobinostat increased to only 12.1%. After the addition of 3% formaldehyde, approximately 80% of the progenies were apoptotic in JMML. An apparent difference in panobinostat-mediated apoptosis between HL60 cells and JMML $CD34^+$ cells was also observed after BrdU/7-AAD staining (Fig. 3B). Exposure to panobinostat decreased percentage of S-phase cells by approximately 20% in HL60 cells. Meanwhile, S-phase arrest was negligible or modest in JMML $CD34^+$ cells.

A great majority of day 7-progenies generated from JMML $CD34^+$ cells under stimulation with SCF+TPO on AGM-S3 cells did not react with mAb against CD14 and CD15 antigens in the three groups (treatment with no reagent, 10 nM of panobinostat or 4 μM of 5-azacytidine).

Effects of panobinostat or 5-azacytidine on the growth of progenies with abnormal karyotype

We examined whether karyotypic change was found by the addition of panobinostat

or 5-azacytidine to the culture, using FISH analysis. $CD34^+$ cells of three JMML patients with either monosomy 7 or trisomy 8 were cultured in the presence or absence of the inhibitor for 14 days and/or 28 days. When compared with the value obtained with no reagent, the addition of panobinostat at 10 nM or 5-azacytidine at 4 μM failed to affect the frequency of the progenies with abnormal karyotype except for the effect of panobinostat in Patient 5 (Fig. 4).

Effects of panobinostat or 5-azacytidine on the propagation of $CD34^+$ cells from healthy donors under stimulation of SCF+TPO on AGM-S3 cells

Finally, we evaluated effects of panobinostat or 5-azacytidine on the propagation of $CD34^+$ cells from healthy donors under stimulation of SCF+TPO on AGM-S3 cells. As shown in Fig. 5A, numbers of day 7-CB $CD34^+$ cells were significantly reduced by the addition of panobinostat at concentrations of 10 nM or higher. However, the addition of 5-azacytidine (2 μM and 4 μM) resulted in modest decline or increase of $CD34^+$ cell production (Fig. 5B). Similar results were obtained by bone marrow $CD34^+$ cells.

Discussion

In the present study, panobinostat at concentrations of 10 nM and 20 nM significantly reduced the numbers of day 7-$CD34^+$ cells that were generated under stimulation with SCF+TPO on irradiated AGM-S3 cells in all eight patients with JMML who possessed *PTPN11* mutation with or without trisomy 8, *NRAS* mutation with or without monosomy 7, *CBL* mutation or monosomy 7. At 40 nM of the inhibitor, almost complete inhibition was achieved. Estimated IC_{50} values of the inhibitor were 9.2 ± 3.0 nM for JMML $CD34^+$ cells. Savelieva *et al* revealed that the mean Cmax of 581 patients with advanced hematologic and solid tumors who were treated with 20 mg panobinostat was 28.5 ± 3.5 nM [17]. According to a phase I study of oral once daily panobinostat (20 mg) in Japanese adult patients with advanced solid tumors, the mean Cmax on day 1 was 30.9 ± 8.6 nM [18]. Thus, the inhibitor may be effective when used clinically for patients with JMML. Panobinostat at the concentrations of 10 nM and 20 nM suppressed the growth of JMML $CD34^+CD38^-$ cell population to a significantly greater extent than that of $CD34^+CD38^+$ cell population. According to FISH analysis, there was no substantial difference in the frequency of day 14- and day 28-progenies with karyotypic abnormality generated from $CD34^+$ cells in the presence or absence of

panobinostat except Patient 5. In this patient, 95% of the progenies showed 7 monosomy, and the remaining 5% of them displayed 7 disomy after exposure to panobinostat, being statistically different from the results obtained from no reagent according to interphase FISH. Nevertheless, the difference was markedly smaller than the value reported previously [15]. One possible explanation is that 2 signals per nucleus may be due in part to the presence of cells with 4N. Panobinostat failed to influence the ability of AGM-S3 cells to stimulate JMML $CD34^+$ cell production. Accordingly, panobinostat may directly inhibit the growth of JMML $CD34^+$ cells regardless of genetic abnormality type. In addition, the HDAC inhibitor appears to exert suppressive effects on JMML stem cells more than more mature progenitors. Considering from the findings reported by Caye et al. [5], there may be a possible role in the PRC2 complex implicated in the switches of methylation and acetylation status of histone H3 lysine 27. Further study will be required.

Panobinostat also suppressed the SCF+TPO-dependent proliferation of CB and normal BM $CD34^+$ cells on AGM-S3 cells, while it remains unclear that the HDAC inhibition acts in the same way to block the proliferation of JMML and normal $CD34^+$

cells. The most common toxicities of HDAC inhibitors including panobinostat are thrombocytopenia and neutropenia in addition to diarrhea, nausea, vomiting and fatigue [19] [20]. Our in vitro culture results may explain the hematologic toxicities seen in the clinical studies. Taken together, panobinostat may be a useful anti-leukemic drug to target JMML stem cells at a pre-transplant stage rather than at a post-transplant stage.

Only approximately 10% of the progenies generated from JMML $CD34^+$ cells under stimulation with SCF+TPO in the presence of 20 nM of panobinostat on AGM-S3 cells were apoptotic according to annexin V/PI staining and BrdU/7-AAD staining. On the contrary, a large part of HL60 cells treated with panobinostat were positive for annexin V and/or PI, being consistent with those reported by Qi *et al* [21]. Furthermore, S-phase arrest was negligible or modest in JMML $CD34^+$ cells. HL60 cells revealed a marked proliferation, whereas an increase of JMML $CD34^+$ cells was much lower (cell numbers after 2 days of culture were 3.27-fold in HL60 cells, and 1.08-fold in JMML $CD34^+$ cells). Thus, there may be no relevant apoptosis and cell cycle block in the suppression of panobinostat on growth of JMML $CD34^+$ 7 cells.

In the present study, the significant inhibitory effects of 5-azacytidine on the growth

of JMML $CD34^+$ cells were not observed, when compared with the results of panobinostat. This may be not resulted from degradation of 5-azacytidine in culture. First, the reagent failed to influence the proliferation of JMML $CD34^+$ cells when 4 μM of 5-azacytidine was added every three days up to day 14. Second, culture supernatant obtained 7 days after incubation of HL60 cells with 4 or 8 μM of 5-azacytidine inhibited the growth of HL60 cells to a similar extent to the value achieved by the addition of fresh 5-azacytidine (4 or 8 μM). In the present study, we used dimethyl sulfoxide to dissolve the reagent. Meanwhile, the other investigators used water [22],[23]. The different procedure of dissolving the reagent may influence 5-azacytidine activity. A group of Niemeyer *et al* reported a clinical response of azacytidine in JMML [9],[24]. Based on our results, the clinical effects of 5-azacytidine may be a result of indirect effects on JMML $CD34^+$ cell proliferation.

Conflict of interest

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1 Effects of panobinostat or 5-azacytidine on the proliferation of JMML $CD34^+$ PB cells

PB $CD34^+$ cells (0.4 to 2 × 10^4/well) of 8 patients with JMML were cultured in wells containing SCF+TPO with or without different concentrations of panobinostat or 5-azacytidine on irradiated AGM-S3 cells. (A) Expression of CD38 antigen on day 7-cultured JMML $CD34^+$ cells generated in wells containing SCF+TPO with or without panobinostat (10 nM, 20 nM) or 5-azacytidine (4 μM, 8 μM) on AGM-S3 cells were evaluated according to flow cytometric analysis. The results shown were derived from one representative experiment out of eight. (B) The numbers of $CD34^+$ cells, $CD34^+CD38^-$ cells, and $CD34^+CD38^+$ cells after 7 days of culture were presented. PB $CD34^+$ cells isolated from 8 patients with JMML expanded to approximately 9.1 ± 7.8 (1.7 to 22.6)-fold the initial number after 7 days of culture. The values are expressed as

a percentage of the cell number obtained in the control culture (no reagent).

Significantly different from no agent: $^{*}p<0.001$, $^{\#}p<0.05$. NS, not significant.

Significant difference between $CD34^{+}CD38^{-}$ cells and $CD34^{+}CD38^{+}$ cells: $^{\dagger}p<0.01$, $^{\#}$ $p<0.05$. NS, not significant.

Figure 2 Effects of panobinostat on the stimulatory ability of AGM-S3 cells to generate JMML $CD34^{+}$ cells

Irradiated confluent AGM-S3 cells were cultured with or without panobinostat (40 nM) for 7 days, and then JMML $CD34^{+}$ cells were seeded with SCF+TPO on the stromal cells rinsed off the inhibitor with the culture medium. 5-azacytidine was used at a concentration of 8 μM. The numbers of day 7-cultured $CD34^{+}$ cells, $CD34^{+}CD38^{-}$ cells, and $CD34^{+}CD38^{+}$ cells were evaluated.

Figure 3 Effects of panobinostat on apoptosis and cell cycle of progenies generated from $CD34^{+}$ cells on AGM-S3 cells in the presence of SCF+TPO

JMML $CD34^{+}$ cells were cultured in wells containing SCF+TPO with or without panobinostat (20 nM) or 5-azacytidine (4 μM) on irradiated AGM-S3 cells. Two days later, cell cycle distribution was examined, using annexin V/PI (A) and BrdU/7-AAD

(B). HL60 cells were used as control cells. ND, not done.

Figure 4 Effects of panobinostat or 5-azacytidine on the growth of progenies with abnormal karyotype

$CD34^+$ PB cells (2 x 10^4/well) of 3 patients with JMML were cultured in wells containing SCF+TPO with or without panobinostat (10 nM) or 5-azacytidine (4 μM) on irradiated AGM-S3 cells. After 14 and 28 days of culture, the frequency of progenies with abnormal karyotype was evaluated according to FISH analysis.

Figure 5 Effects of panobinostat or 5-azacytidine on the propagation of CB $CD34^+$ cells under stimulation of SCF+TPO on AGM-S3 cells

$CD34^+$ cells (2 x 10^4/well) from 3 CB samples (CB 1, black; CB 2, white; CB 3, gray) were cultured in wells containing SCF+TPO with or without different concentrations of panobinostat on irradiated AGM-S3 cells. 5-azacytidine was added at the concentrations ranging from 2 μM to 8 μM. The numbers of $CD34^+$ cells, $CD34^+CD38^-$ cells, and $CD34^+CD38^+$ cells were evaluated after 7 days of culture. The values are expressed as a percentage of the cell number obtained in the control culture (no reagent). ND, not done. Significantly different from no reagent: $^*p<0.0001$, ¶

$p<0.001$, $^{\dagger}p<0.01$, $^{\#}p<0.05$. NS, not significant.

Supplementary Figure S1 Effects of panobinostat or 5-azacytidine on the growth of HL-60 and THP-1 cells

HL60 cells (■) and THP-1 cells (□) were cultured at 1 x 10^5 cells per well with or without different concentrations of panobinostat or 5-azacytidine. On day 3, the numbers of HL60 cells and THP-1 cells were increased to 3.3 x 10^5 cells and 7.8 x 10^5 cells, respectively, in wells containing no agent. The values are expressed as a percentage of the cell number obtained in the control culture (no reagent). Significantly different from no panobinostat: *$p<0.0001$.

Supplementary Figure S2 Effect of culture supernatant obtained 7 days after incubation of HL60 cells with 4 or 8 μM of 5-azacytidine on the growth of HL60 cells

HL60 cells were cultured in the presence or absence of 5-azacytidine (4 or 8 μM). After 7 days, numbers of HL60 cells per well were $3.5 \pm 0.2 \times 10^5$ in the absence of the reagent, $2.4 \pm 0.5 \times 10^4$ in 4 μM of 5-azacytidine, and $3.3 \pm 0.3 \times 10^3$ in 8 μM of

5-azacytidine. The individual culture supernatant was obtained after centrifugation. Inhibitory potential of the culture supernatants (white bars) on the growth of HL60 cells was compared with that of fresh 5-azacytidine (4 or 8 μM, black bars). The values are expressed as a percentage of the cell number obtained in the control culture (no reagent). Significantly different from no reagent: * $p<0.0001$. NS, not significant.

Supplementary Figure S3 Effects of panobinostat and 5-azacytidine on mature population generated from JMML $CD34^+$ cells

Because of low frequencies of $CD34^-CD38^+$ cells and $CD34^-CD38^-$ cells, the two groups were combined as $CD34^-$ cells. Significantly different from no reagent: $^*p<0.0001$. NS, not significant.

Table 1 Genetic and chromosomal characteristics of 8 children with JMML

	Sex	Age	*PTPN11*	*NRAS*	*CBL*	*SETBP1*	*JAK3*	Karyotype
Patient 1	M	3y	c.227A>G	wt	wt	wt	wt	trisomy 8
Patient 2	M	5y	c.227A>G	wt	wt	wt	wt	normal
Patient 3	M	4y	wt	c.37G>C	wt	wt	wt	normal
Patient 4	M	4y	wt	c.38G>A	wt	wt	wt	normal
Patient 5	M	5y	wt	c.182A>C	wt	wt	wt	monosomy 7
Patient 6	M	1m	wt	wt	c.1095-1227del	wt	wt	normal
Patient 7	M	2m	wt	wt	c.1255T>C	wt	wt	normal
Patient 8	M	1y	wt	wt	wt	wt	wt	monosomy 7

Abbreviation: m, months; wt, wild-type nucleotide in the region analyzed for each gene; y, years

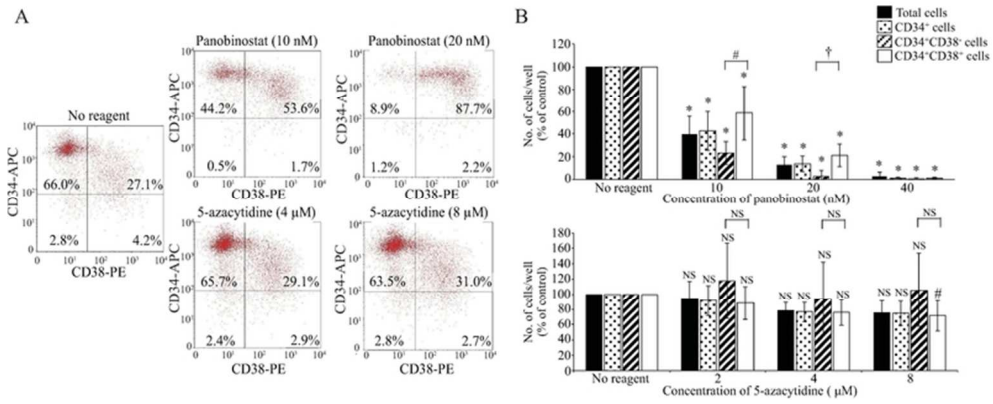

Figure 1 Effects of panobinostat or 5-azacytidine on the proliferation of JMML CD34+ PB cells‼ ┼ PB CD34+ cells (0.4 to 2 × 104/well) of 8 patients with JMML were cultured in wells containing SCF+TPO with or without different concentrations of panobinostat or 5-azacytidine on irradiated AGM-S3 cells. (A) Expression of CD38 antigen on day 7-cultured JMML CD34+ cells generated in wells containing SCF+TPO with or without panobinostat (10 nM, 20 nM) or 5-azacytidine (4 μM, 8 μM) on AGM-S3 cells were evaluated according to flow cytometric analysis. The results shown were derived from one representative experiment out of eight. (B) The numbers of CD34+ cells, CD34+CD38- cells, and CD34+CD38+ cells after 7 days of culture were presented. PB CD34+ cells isolated from 8 patients with JMML expanded to approximately 9.1 ± 7.8 (1.7 to 22.6)-fold the initial number after 7 days of culture. The values are expressed as a percentage of the cell number obtained in the control culture (no reagent). Significantly different from no agent: *p<0.001, # p<0.05. NS, not significant. Significant difference between CD34+CD38- cells and CD34+CD38+ cells: † p<0.01, # p<0.05. NS, not significant.‼ ┼

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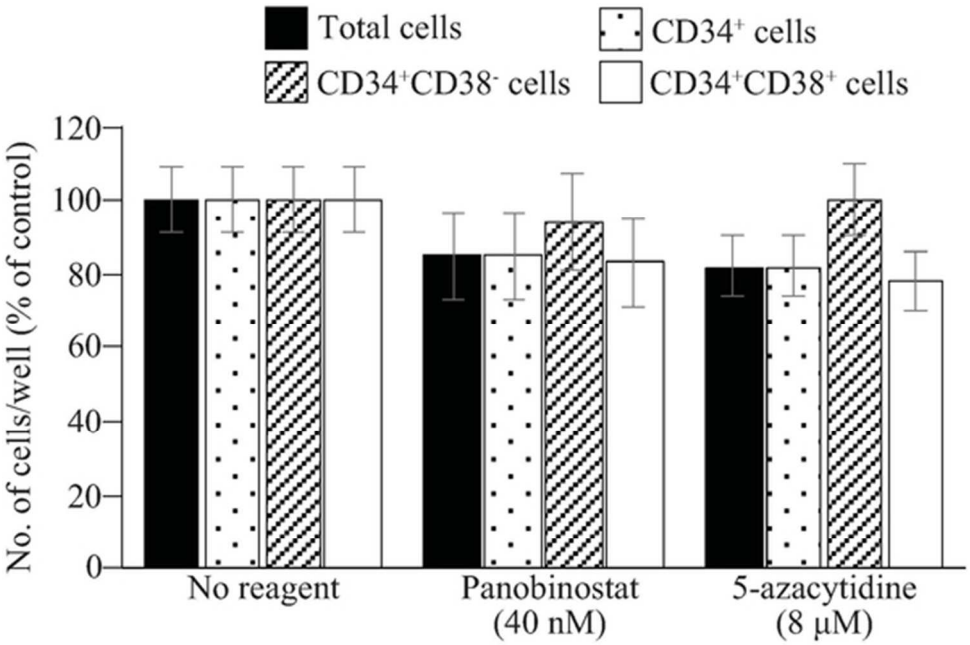

Figure 2 Effects of panobinostat on the stimulatory ability of AGM-S3 cells to generate JMML CD34+ cells
Irradiated confluent AGM-S3 cells were cultured with or without panobinostat (40 nM) for 7 days, and then JMML CD34+ cells were seeded with SCF+TPO on the stromal cells rinsed off the inhibitor with the culture medium. 5-azacytidine was used at a concentration of 8 μM. The numbers of day 7-cultured CD34+ cells, CD34+CD38- cells, and CD34+CD38+ cells were evaluated.

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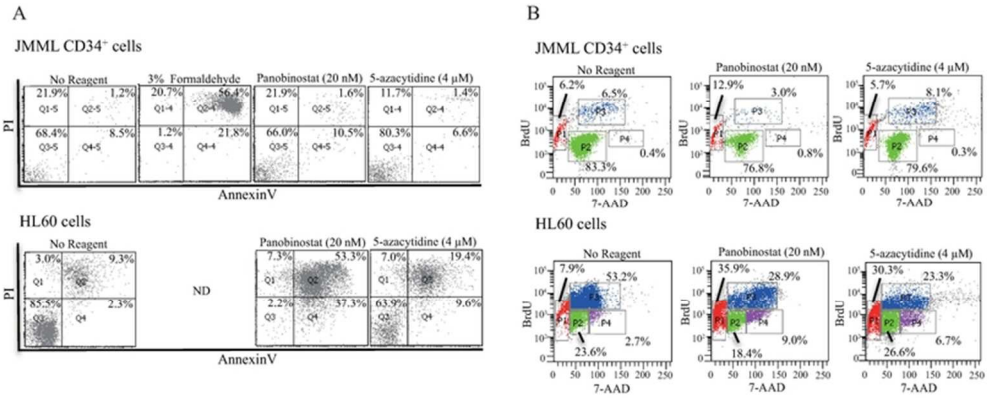

Figure 3 Effects of panobinostat on apoptosis and cell cycle of progenies generated from CD34+ cells on AGM-S3 cells in the presence of SCF+TPO‼ ┼ JMML CD34+ cells were cultured in wells containing SCF+TPO with or without panobinostat (20 nM) or 5-azacytidine (4 μM) on irradiated AGM-S3 cells. Two days later, cell cycle distribution was examined, using annexin V/PI (A) and BrdU/7-AAD (B). HL60 cells were used as control cells. ND, not done.‼ ┼

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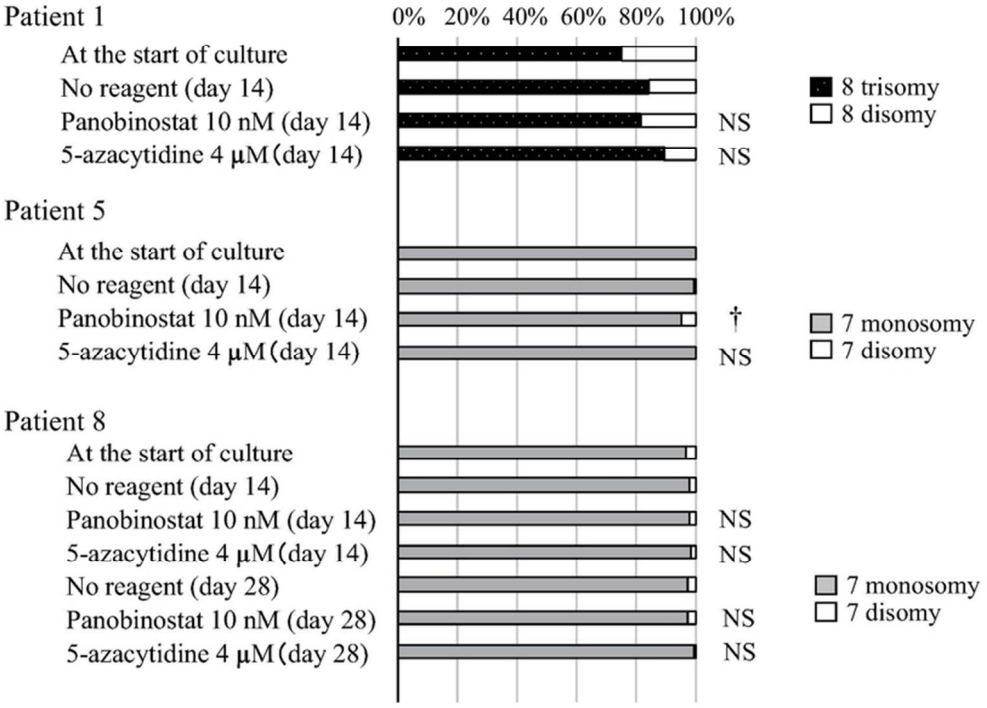

Figure 4 Effects of panobinostat or 5-azacytidine on the growth of progenies with abnormal karyotype CD34+ PB cells (2 x 104/well) of 3 patients with JMML were cultured in wells containing SCF+TPO with or without panobinostat (10 nM) or 5-azacytidine (4 μM) on irradiated AGM-S3 cells. After 14 and 28 days of culture, the frequency of progenies with abnormal karyotype was evaluated according to FISH analysis.

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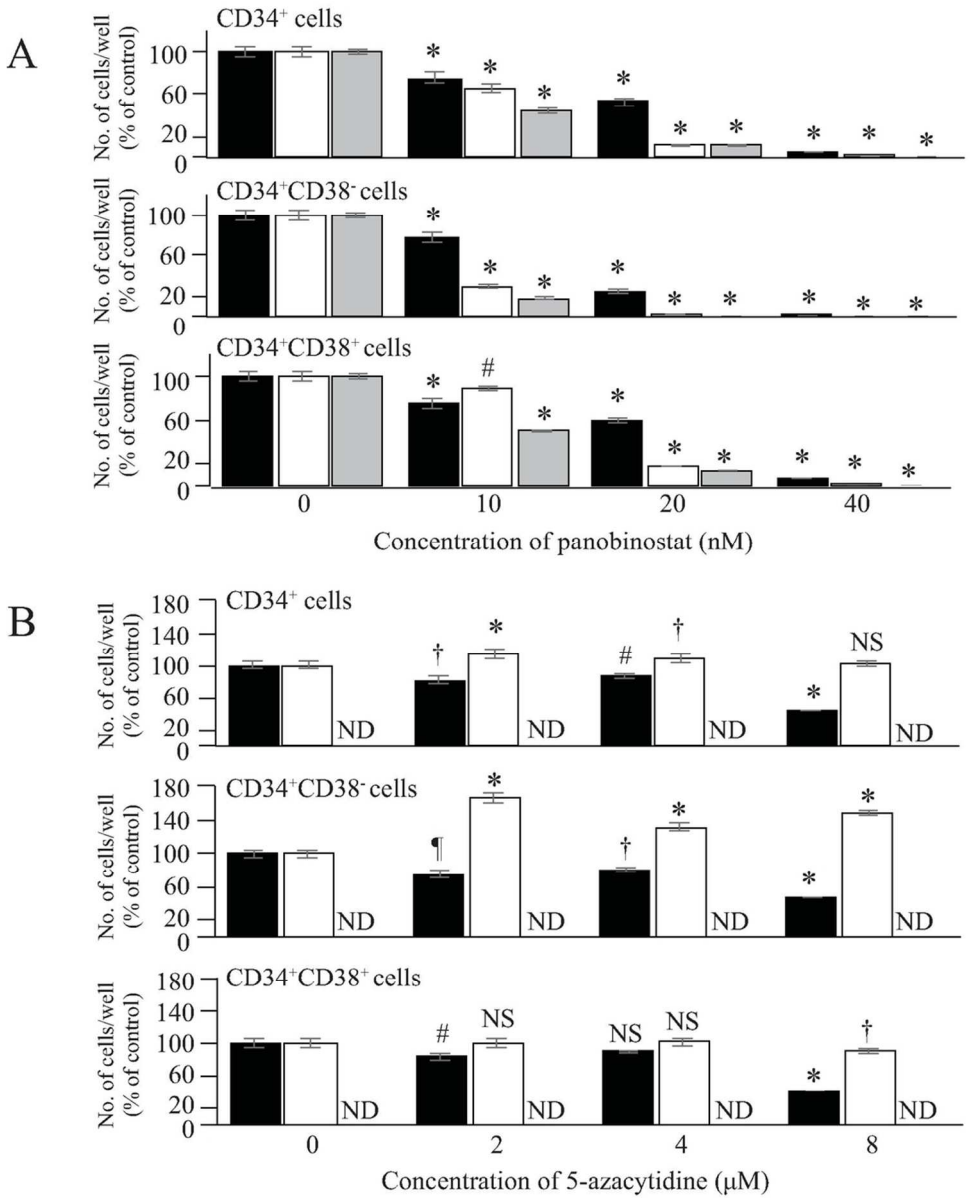

Figure 5 Effects of panobinostat or 5-azacytidine on the propagation of CB CD34+ cells under stimulation of SCF+TPO on AGM-S3 cells

CD34+ cells (2 x 104/well) from 3 CB samples (CB 1, black; CB 2, white; CB 3, gray) were cultured in wells containing SCF+TPO with or without different concentrations of panobinostat on irradiated AGM-S3 cells. 5-azacytidine was added at the concentrations ranging from 2 μM to 8 μM. The numbers of CD34+ cells, CD34+CD38- cells, and CD34+CD38+ cells were evaluated after 7 days of culture. The values are expressed as a percentage of the cell number obtained in the control culture (no reagent). ND, not done. Significantly different from no reagent: *p<0.0001, ¶ p<0.001, † p<0.01, # p<0.05. NS, not significant.

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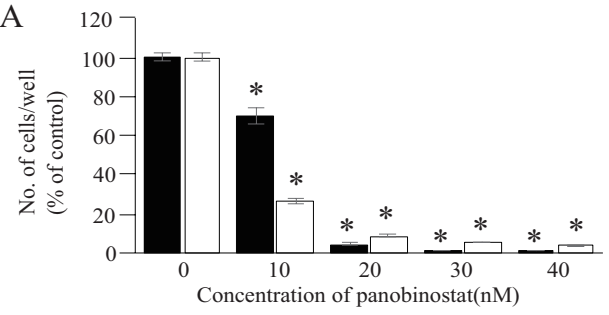
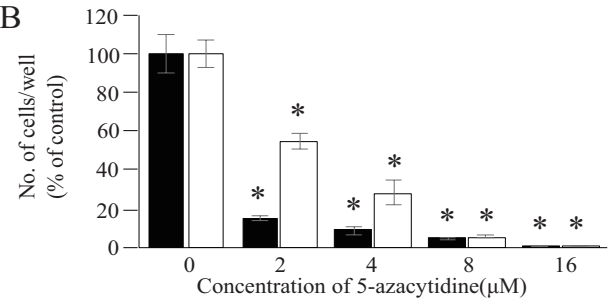
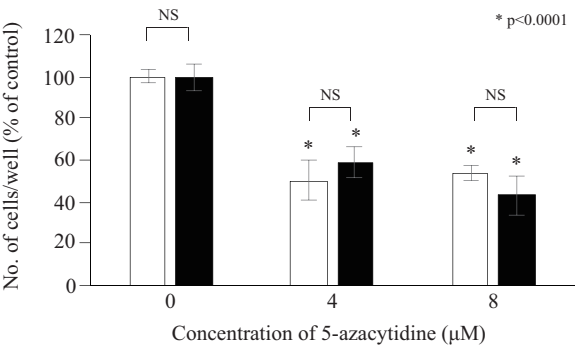

A
120
100
80
60
40
20
0
No. of cells/well
(% of control)
*
*
*
*
*
*
*
*
0
10
20
30
40
Concentration of panobinostat(nM)


B
120
100
80
60
40
20
0
No. of cells/well
(% of control)
*
*
*
*
*
*
*
*
0
2
4
8
16
Concentration of 5-azacytidine(μM)


NS
* p<0.0001
NS
NS
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40
20
0
No. of cells/well (% of control)
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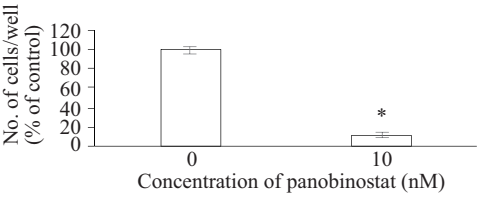
4
8
Concentration of 5-azacytidine (μM)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*
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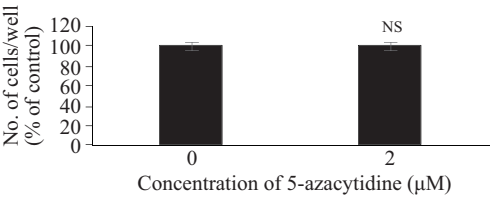
10
Concentration of panobinostat (nM)


No. of cells/well (% of control)
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100
80
60
40
20
0
NS
0
2
Concentration of 5-azacytidine (μM)