HAR cells. The results shown are representative of three independent experiments. (B) (C) Cells were treated with azacitidine for 24 (B) or 12 (C) hours. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading.

Figure 3. **Azacitidine-mediated changes in DNMT activity aud *p16* mRNA levels are abrogated in azacitidine-resistant cells.** Cells were cultured in the indicated concentrations of azacitidine for 24 hours. (A) Nuclear extracts were prepared from the cells. DNMT activity was determined using DNMT activity/inhibition assay system (ActiveMotif, Carlsbad, CA). The results are expressed as ratio of azacitidine-treated cells to non-treated cells. Statistical analysis was carried out using Student's t-test for comparison of the data between untreated cells and cells treated with azacitidine. (B) Total cell lysates were prepared from the cells and subjected to Western blot analysis using antibodies against DNMT1, DNMT3a and DNMT3b. The expression of *GAPDH* is shown as an internal control. (C) Total RNA was prepared from the cells. The levels of *p16* mRNA were determined by real-time PCR analyses using cDNA, which was generated from total RNA extracted from each cell line. *GAPDH* cDNA was used as an internal control. The results were calculated using the DDC_T method and are expressed as the ratio of *p16* mRNA level in cells treated with azacitidine to that in untreated cells. Statistical analysis was carried out using Student's t-test for comparison of the data between untreated cells and cells treated with azacitidine.

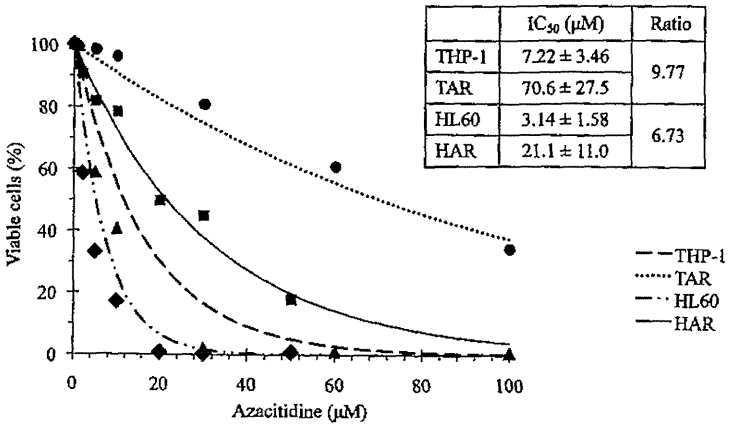
Figure 4. **The level of equilibrative nucleoside transporters.** (A) Expression of nucleoside transporters in THP-1, TAR, HL60 and HAR cells was evaluated by quantitative real-time PCR. The amounts of hENT1, hENT2 and hCNT1 mRNA relative to that of GAPDH were determined as described in "Materials and Methods". Statistical analysis was carried out using Student's t-test for comparison of the data between azacitidine-resistant cells and their corresponding parental cells. (B)

Expression of p-glycoprotein in THP-1, TAR, HL60 and HAR cells was determined by flow cytometry as described in "Materials and Methods".

Figure 5. ***UCK2* gene mutations are present in azacitidine-resistant cells.** (A) There is no difference in UCK2 protein levels between azacitidine-resistant and the corresponding parental cells. Total cell lysates were prepared and subjected to Western blot analysis using antibodies against UCK2. The expression of *GAPDH* is shown as an internal control. (B) Direct sequence analysis of the *UCK2* gene revealed point mutations in exon 4 and exon 5. (C) IC_{50} values of azacitidine were determined as described in "Materials and Methods". Results are expressed as the ratio of IC_{50} value of cells transfected with PLL3.7 or each *UCK2* expression vector to that of untreated cells. Statistical analysis was carried out using Student's t-test for comparison of the data between untreated cells and cells transfected with PLL3.7 or each *UCK2* expression vector. UCK2mut: expression vector of mutated *UCK2*. UCK2wt: expression vector of wild type *UCK2*. (D) Cells transfected with the indicated expression vectors were treated with azacitidine for 24 hours. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading. (E) Cells transfected with PLL3.7 or each *UCK2* expression vector were cultured in the absence or presence of indicated concentrations of azacitidine for 24 hours. Total RNA was prepared from the cells and the levels of *p16* mRNA were determined by real-time PCR analyses. *GAPDH* cDNA was used as an internal control. The results were calculated using the DDC_T method and are expressed as the ratio of *p16* mRNA level in cells treated with azacitidine to that in untreated cells. Statistical analysis was carried out using Student's t-test for comparison of the data between untreated cells and cells treated with azacitidine.

Figure 6. **siRNA-mediated knockdown of BCL2L10 resulted in no restoration of azacitidine-sensitivity in TAR and HAR cells.** (A) Cells were transfected with BCL2L10 siRNA as described in Materials and Methods. Total cell lysates were

prepared from the cells and subjected to Western blot analysis using antibody against human BCL2L10. The expression of GAPDH is shown as an internal control. (B) Cells were transfected with BCL2L10 siRNA and cultured for 24 hours prior to treatment with azacitidine. After addition of azacitidine, further incubation was performed for 96 hours and IC_{50} values of azacitidine were determined.



	IC_{50} (μM)	Ratio
THP-1	7.22 ± 3.46	9.77
TAR	70.6 ± 27.5	
HL60	3.14 ± 1.58	6.73
HAR	21.1 ± 11.0	

Figure 1A

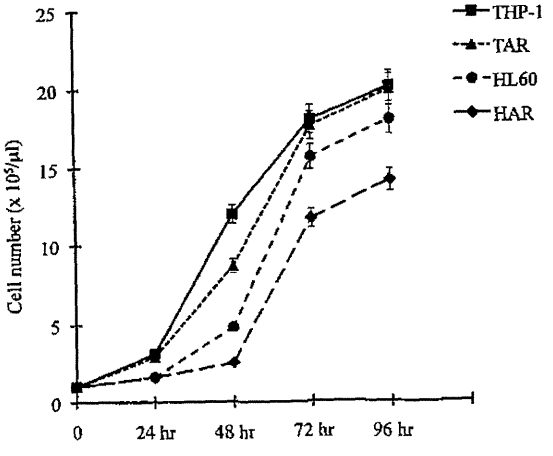


Figure 1B

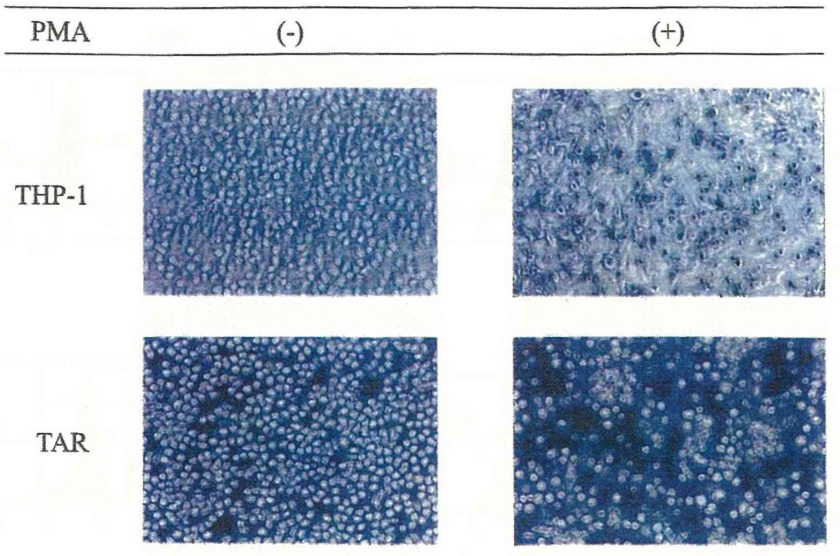


Figure 1C

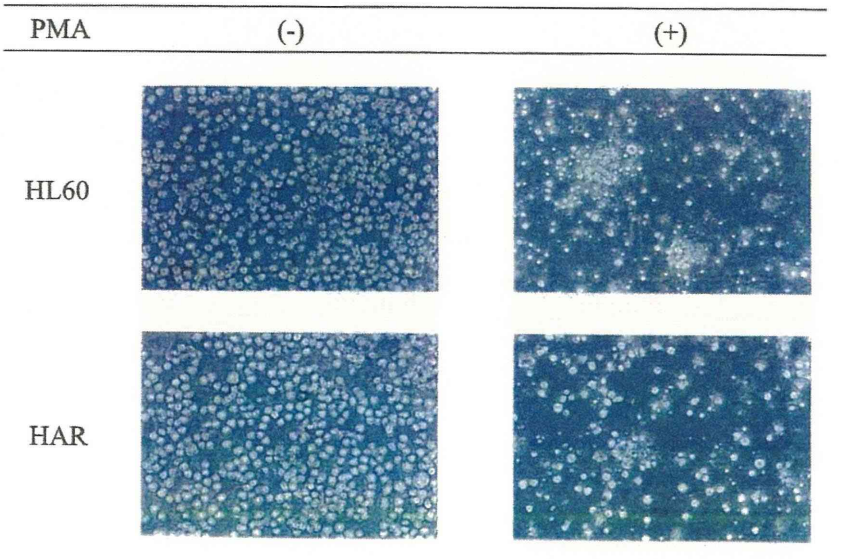


Figure 1C (continued)

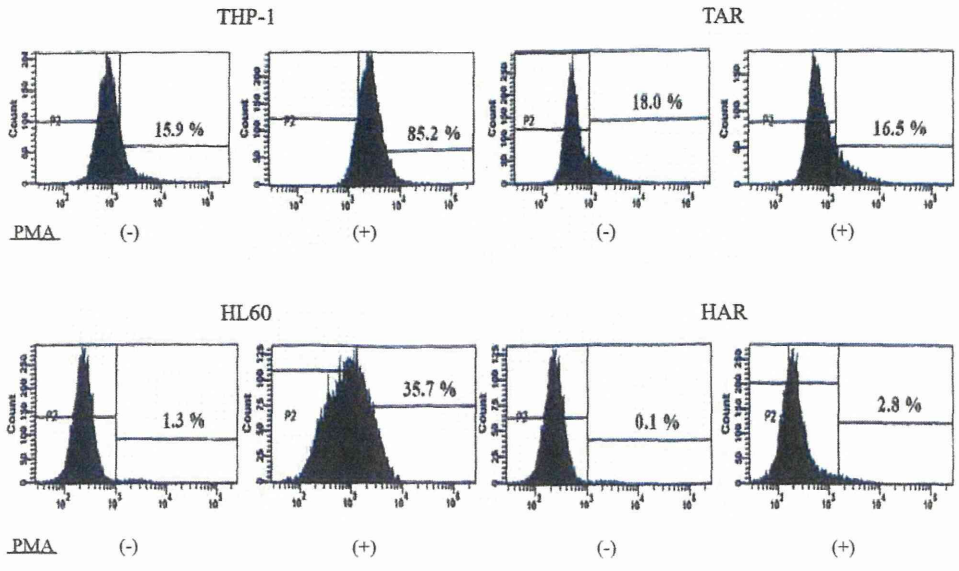


Figure 1D

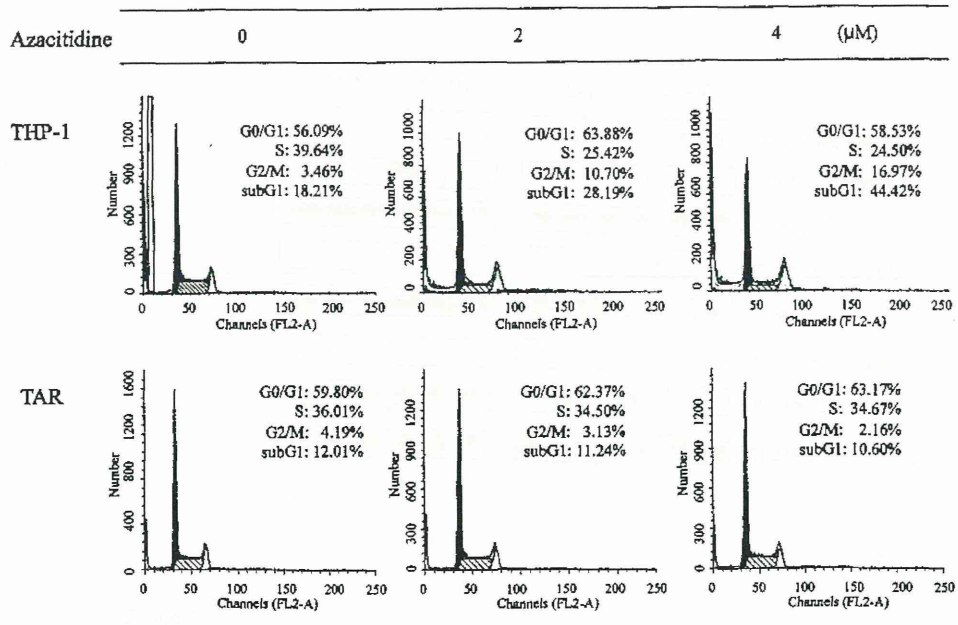


Figure 2A

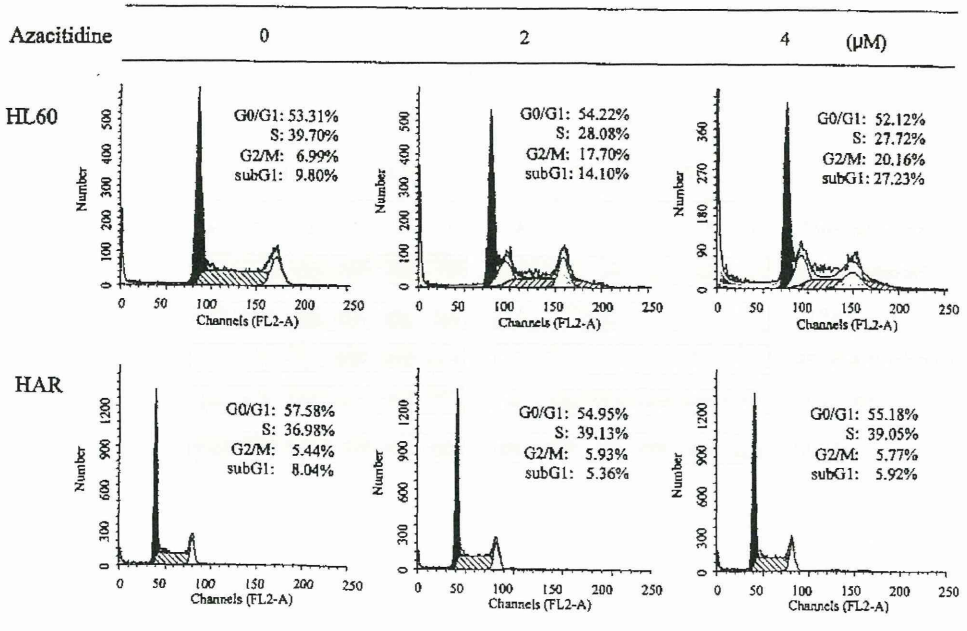


Figure 2A (continued)

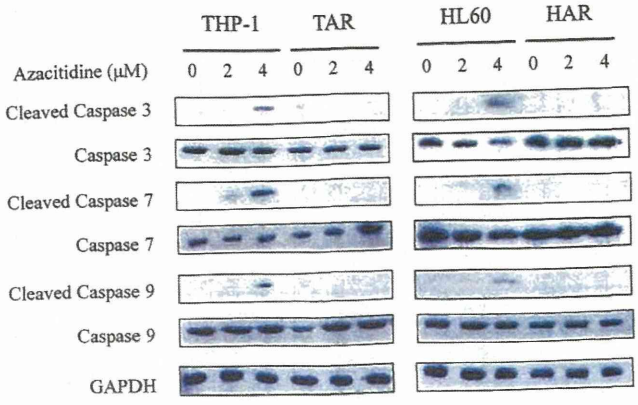


Figure 2B

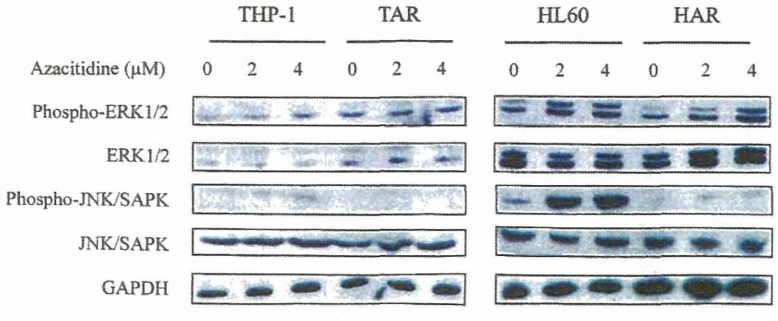
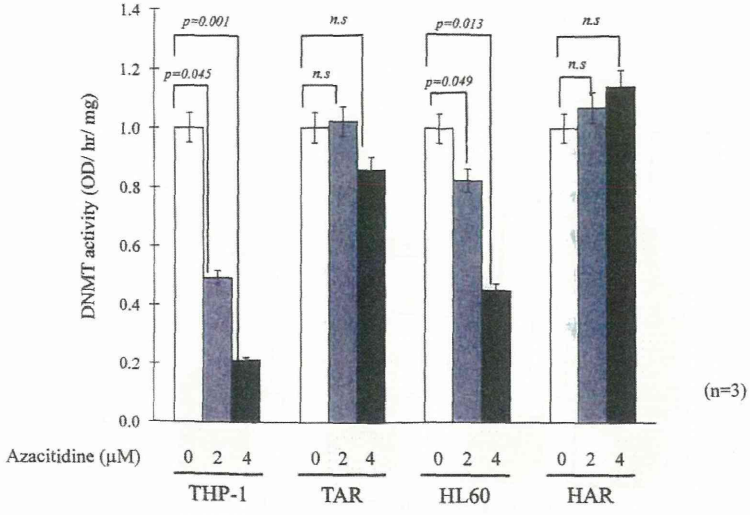


Figure 2C



(n=3)

Figure 3A

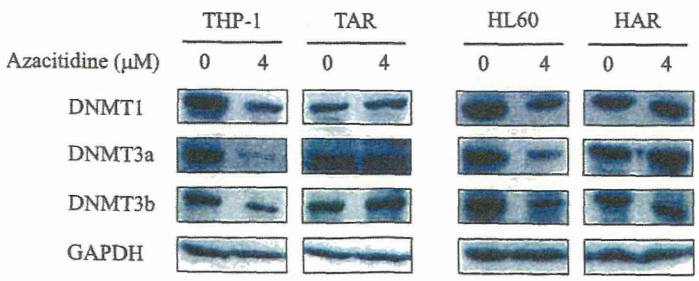
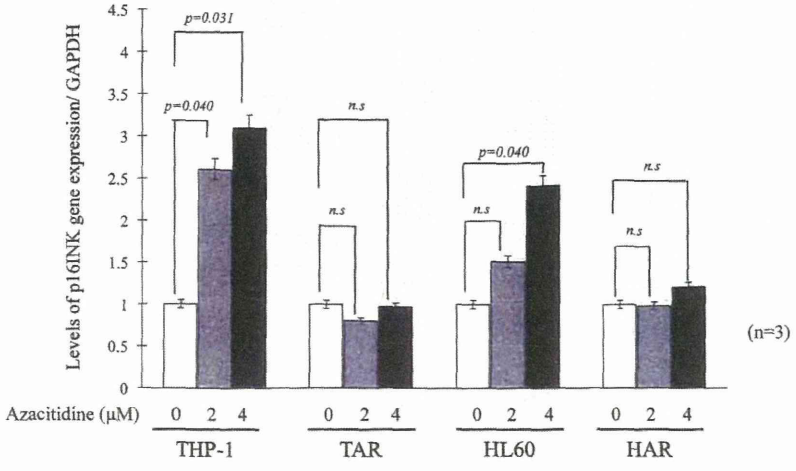


Figure 3B


Levels of p16INK gene expression/ GAPDH
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
p=0.031
p=0.040
n.s
n.s
p=0.040
n.s
n.s
n.s
Azacitidine (μM)
0 2 4
0 2 4
0 2 4
0 2 4
THP-1
TAR
HL60
HAR

(n=3)

Figure 3C

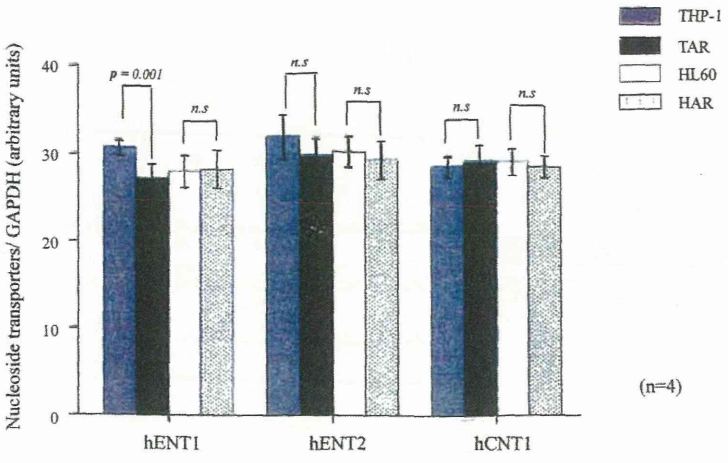


Figure 4A

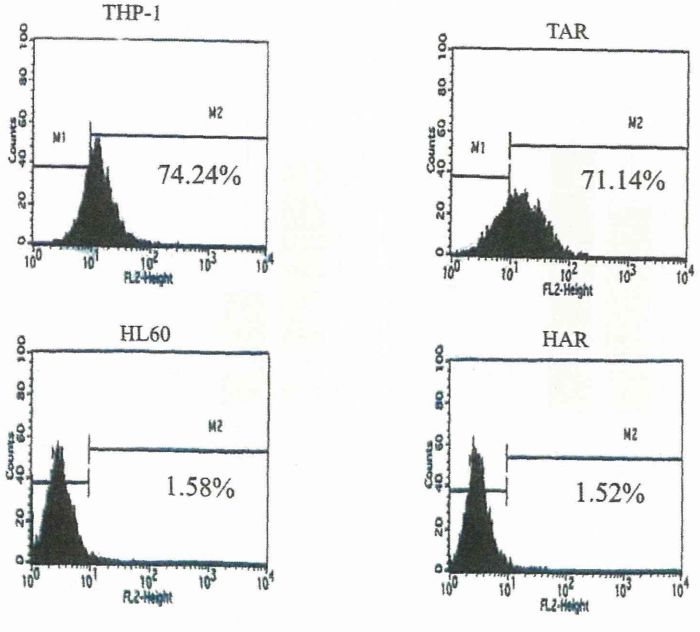


Figure 4B

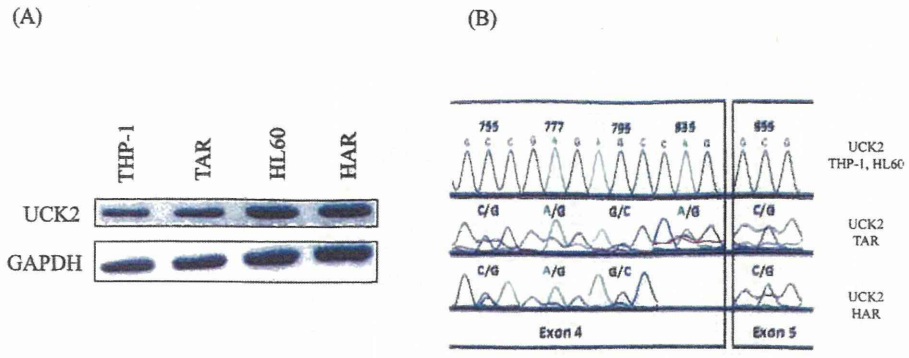


Figure 5

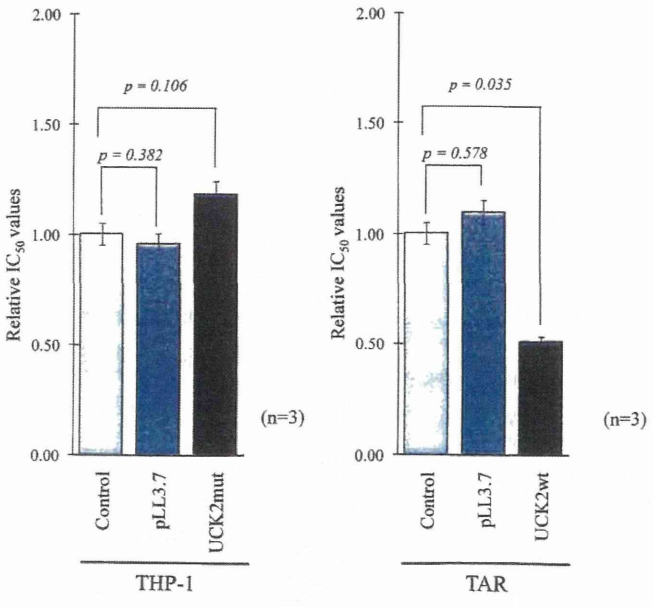


Figure 5C

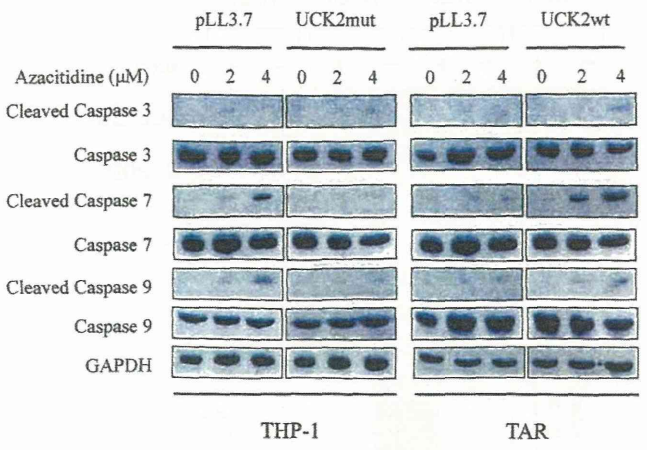


Figure 5D

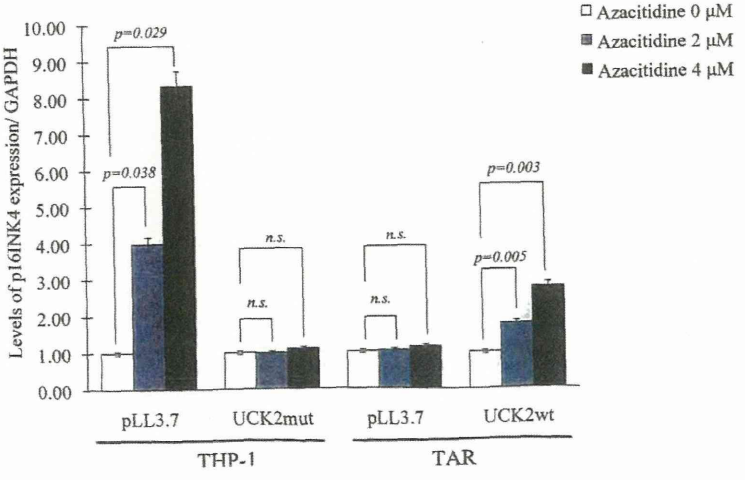
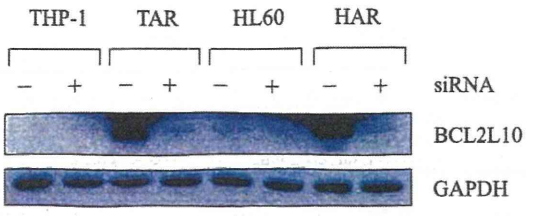


Figure 5E

(A)


THP-1
TAR
HL60
HAR
− + − + − + − +
siRNA
BCL2L10
GAPDH

(B)

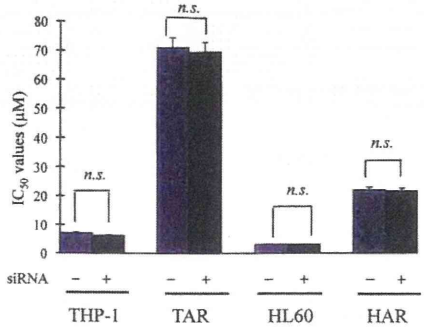

n.s.
n.s.
n.s.
n.s.
IC50 values (μM)
80
70
60
50
40
30
20
10
0
siRNA
− + − + − + − +
THP-1
TAR
HL60
HAR

Figure 6

Supplementary Table 1. Primers used for cDNA amplifications

Genes	Forward	Reverse	Position
DNMT3a	5'-acgacagcgatgagagtgac-3'	5'-cccaatcaccagatcgaatg-3'	
DNMT3b-1	5'-aggaaagcatgaagggagacac-3'	5'-cagctggtcctccaatgagtct-3'	314 - 1,308
DNMT3b-2	5'-atttggccaccttcaataagctc-3'	5'-ttcctcacgtcgttcacgtattt-3'	1,184 - 2,150
DNMT3b-3	5'-cgcttctgaagtgtgtgaggagt-3'	5'-gtagtgcacaggaaagccaaaga-3'	2,067 - 2,706
DNMT1-1	5'-gagatgccggcgcgtaccgc-3'	5'-gtgggtgctgcccatatttga-3'	178 - 1,342
DNMT1-2	5'-ccaacggagaaaaaaatggct-3'	5'-tcccctggtgcatttttttgg-3'	1,231 - 2,401
DNMT1-3	5'-agcaagcaggcttgccaagag-3'	5'-cgcactcgggcaggtcctccc-3'	2,281 - 3,451
DNMT1-4	5'-ccagcgagctaccacgcagac-3'	5'-ccaccaatgcactcatgtcct-3'	3,331 - 4,501
DNMT1-5	5'-tcggcactggagatctcctac-3'	5'-ttttggtttataggagagatttatttg-3'	4,372 - 5,403
UCK2	5'-gcgaaccatggccggggacagcgag-3'	5'-acagtatgtacagatgagcagtgcc-3'	

Supplementary Table 2. Primers used for sequence analysis

Genes	Forward	Reverse	Position
DNMT3a	5'-gctttctggagtgtgcgtac-3'	5'-cccaatcaccagatcgaatg-3'	
DNMT3b-1	5'-aggaaagcatgaagggagacac-3'	5'-agctcgcaccctagctttct-3'	314 - 1,308
DNMT3b-2	5'-atttggccaccttcaataagctc-3'	5'-ggttccaacagcaatggact-3'	1,184 - 2,150
DNMT3b-3	5'-cgcttctgaagtgtgtgaggagt-3'	5'-gagctcagtgcaccacaaaa-3'	2,067 - 2,706
DNMT1-1	5'-gagatgccggcgcgtaccgc-3'	5'-agggtcgtccaggtactgc-3'	178 - 1,342
DNMT1-2	5'-ccaacggagaaaaaaatggct-3'	5'-cggcatctctgggatgttat-3'	1,231 - 2,401
DNMT1-3	5'-agcaagcaggcttgccaagag-3'	5'-acagccttgaagtccaccac-3'	2,281 - 3,451
DNMT1-4	5'-ccagcgagctaccacgcagac-3'	5'-agatgtggtccctgaggatg-3'	3,331 - 4,501
DNMT1-5	5'-tcggcactggagatctcctac-3'	5'-tttccactcatacagtggtagatttg-3'	4,372 - 5,403
UCK2	5'-cgagcagaccctgcagaac-3'	5'-ccaagagacagaggaggggt-3'	

Supplementary Table 3. Primers used for real-time PCR

Genes	Forward	Reverse
hENT1	5'-tctccaactctcagcccaccaa-3'	5'-cctgcgatgctggacttgacct-3'
hENT2	5'-accatgccctccacctacag-3'	5'-gggcctgggatgatttattg-3'
hCNT1	5'-acctcatagaagcagccagc-3'	5'-ccatcaagaaggagggctacaggc-3'
P16	5'-agccttcggctgactggctgg-3'	5'-ctgcccatcatcatgacctgga-3'

Original Article

Combined azacitidine and romidepsin enhances cytotoxicity in azacitidine-sensitive but not in azacitidine-resistant multiple myeloma cell lines.

Piyanuch Sripayap, Tadashi Nagai, Keiya Ozawa

Division of Hematology, Jichi Medical University,Tochigi, Japan 329-0498

Abstract

In hematopoietic tumor cells, aberrant epigenetic alterations of hypermethylation or histone deacetylation are usually observed. In multiple myeloma (MM), histone deacetylase inhibitors (HDIs) have proven anti-tumor activity, whereas the effects of DNA demethylating agents are obscure. In this study, we examined the effect of DNA demethylating agent azacitidine and HDI romidepsin in human MM cell lines RPMI8226 and U266. In RPMI8226 cells, azacitidine restored *p16* expression accompanied by disruption of its main target molecules DNA methyltransferases (DNMTs), thereby showing anti-tumor effect. However, in U266 cells, azacitidine-mediated demethylation was abrogated, thereby losing its anti-myeloma effect. The combination of azacitidine and romidepsin enhanced induction of apoptosis by activation of the caspase pathway in RPMI8226 cells but not in U266 cells. Furthermore, isobologram analyses showed that this combination had an additive inhibitory effect on the growth of RPMI8226 cells, whereas in U266 cells it had a nearly subtractive effect. These results thus suggest that the combination is effective in azacitidine-sensitive but not in azacitidine-resistant MM cells. Taken together, the results support the utility of this combination as a potential therapy for MM ; however, this therapy should be considered based on the sensitivity of the particular MM cells to azacitidine.

(Keywords : multiple myeloma, azacitidine, romidepsin, DNA demethylating agent, histone deacetylase inhibitor)

Introduction

In hematopoietic tumor cells, aberrant epigenetic alterations due to hypermethylation or overexpression of histone deacetylases are usually observed. These abnormalities result in suppression of the expression of some genes, including tumor suppressor genes and cell-differentiation related genes, thereby they are involved in the pathophysiology of tumor cells (Herman JG, N Engl J Med. 2003. Baylin SB. Nat Clin Pract Oncol. 2005). Therefore, abrogation of aberrant epigenetic states should be a promising therapeutic strategy ; and for this purpose, two DNA demethylating agents azacitidine (5-azacytidine) and decitabine (5-aza-2-deoxycytidine) and several histone deacetylase inhibitors (HDIs) have been developed. DNA demethylating agents incorporate into DNA, resulting in disruption of DNA methyltransferases (DNMTs), thereby suppressing DNA methylation. In addition, azacitidine also incorporates into RNA, developing cytotoxicity by inhibiting protein synthesis (Christman JK. *Oncogene.* 2002 ; 21 : 5483-5495). Both agents are now approved for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) with 20% to 30% bone marrow blasts. HDIs promote the acetylation of histone proteins, subsequently recovering the expression of genes that may be involved in cell cycle regulation, induction of apoptosis and differentiation (Marks PA, J Cell Biochem. 2009). Although the efficacy of HDIs on patients with MDS or AML was not promising when used as a single agent, it has been shown *in vitro* that a combination of HDI and DNA demethylating agent augments anti-tumor activity in leukemia cells.

In multiple myeloma (MM) cells, aberrant epigenetic alterations due to over-expression of histone deacetylases is also usually observed (Stimson L, Ann Oncol. 2009),

Correspondence to : Tadashi Nagai, Division of Hematology, Jichi Medical University, Tochigi, Japan 329-0498, E-mail : t-nagai@jichi.ac.jp
Received : 25 September 2013, Accedpted : 17 October 2013

suggesting that HDIs are potential anti-MM agents. Furthermore, HDIs also affect the acetylation status of non-histone proteins such as heat shock protein 90 and α-tubulin, which are involved in the pathophysiology of MM cells (Bali P, J Biol Chem. 2005). Consequently, the clinical efficacy of HDIs on patients with MM has been investigated (Richardson P, Leuk Lymphoma. 2008, Schmitt S, Onkologie. 2010, Niesvizky R, Cancer. 2011). In contrast, the effects of DNA demethylating agents on MM cells are still obscure.

In this study, we examined the effect of azacitidine on two human MM cell lines and found that the sensitivity to azacitidine is markedly different between the two. We also found that the combination of azacitidine and the HDI romidepsin showed strong enhancement of anti-MM effect in azacitidine-sensitive but not in azacitidine-resistant MM cells.

Materials and Methods

Cell lines—U266 and RPMI8226 are human multiple myeloma cell lines (Drexler HG, Hum Cell. 2003). To determine the IC_{50} values of azacitidine and romidepsin, cells were incubated in the presence of various concentrations of each reagent for 96 hours, then enumerated using a Cell Counting Kit-8 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in accordance with the manufacturer's instructions. Based on the number of cells found, dose response curves were prepared, and concentrations yielding 50% cellular viability were designated as IC_{50}. All cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin G, and streptomycin sulfate ; and split every 4 or 5 days.

Reagents—Azacitidine was purchased from Sigma Chemical Co. (St. Louis, MO). Romidepsin was purchased from Toronto Research Chemicals Inc. (Ontario, Canada).

Western blot analysis—Whole cell lysates were prepared from 1×10^7 cells. Then 30 μg of lysates was separated electrophoretically using 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously (Miyoshi T, Exp Hematol. 2007). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, which was used as an internal control, was purchased from Chemicon International (Temecula, CA). Anti-DNA methyltransferase 1 (DNMT1), anti-DNMT3a and anti-DNMT3b were purchased from ActiveMotif (Carlsbad, CA). Anti-acetyl-histone H3 and anti-acetyl-histone H4 rabbit polyclonal antibodies were purchased from Merck Millipore. (Billerica, MA). Rabbit polyclonal antibodies against caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7, caspase-9, cleaved caspase-9, PARP and cleaved PARP were purchased from Cell Signaling Technology (Beverly, MA).

DNMT activity assay—Cells were cultured in the presence or absence of azacitidine for 24 or 48 hours. Nuclear extracts were then prepared as described previously (Lassar AB, Cell. 1991). DNMT activity was determined using a DNMT activity/inhibition assay system (ActiveMotif, Carlsbad, CA) in accordance with the manufacturer' s instructions.

Real-time PCR analysis—The cDNA was generated from total RNA by Superscript II reverse transcriptase and subjected to PCR with SYBR green. PCR products were analyzed using an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA). Complimentary DNA corresponding to the GAPDH gene was used for the internal control of these real-time analyses. The primers used were 5'-agccttcggctgactggctgg-3' (forward) and 5'-ctgcccatcatcatgacctgga-3' (reverse) for p16. The results were calculated using the DDC_T method.

Cytotoxic effects of the combination of azacitidine and romidepsin—The cytotoxic effects of the combination of azacitidine and romidepsin were evaluated by a Steel and Peckham isobologram as described previously (Nagai T *Leuk Res.* 2010). The basis of the theory and the detailed procedure of this analysis have been described in a previous report (Steel GG, *Int. J. Radiat. Oncol. Biol. Phys.* 1979). Briefly, when the points lie outside the left margin of the envelope formed by two dotted lines, the combination treatment is considered to have a synergistic inhibitory effect on cell growth. In contrast, if the points lie outside the right margin of the envelope, the combination treatment is considered to have an antagonistic effect. If the points lie within the envelope, the combination treatment is considered to have an additive effect.

Results

RPMI8226 cells are sensitive and U266 cells are resistant to azacitidine.

We first examined the growth-inhibitory effects of azacitidine and romidepsin in two human MM cell lines, U266 and RPMI8226. The IC_{50} value of azacitidine against RPMI8226 cells was similar to that against two human leukemia cell lines THP-1 and HL60. However, the IC_{50} value of azacitidine against U266 cells was significantly higher (Figure 1), suggesting that RPMI8226 cells are sensitive and U266 cells are resistant to azacitidine. In contrast, the IC_{50} values of romidepsin against RPMI8226 and U266 cells were slightly lower than those against the leukemia cell lines, indicating that romidepsin has anti-MM effect in both cell lines.

Azacitidine-mediated disruption of DNMT is abrogated in U266 cells.

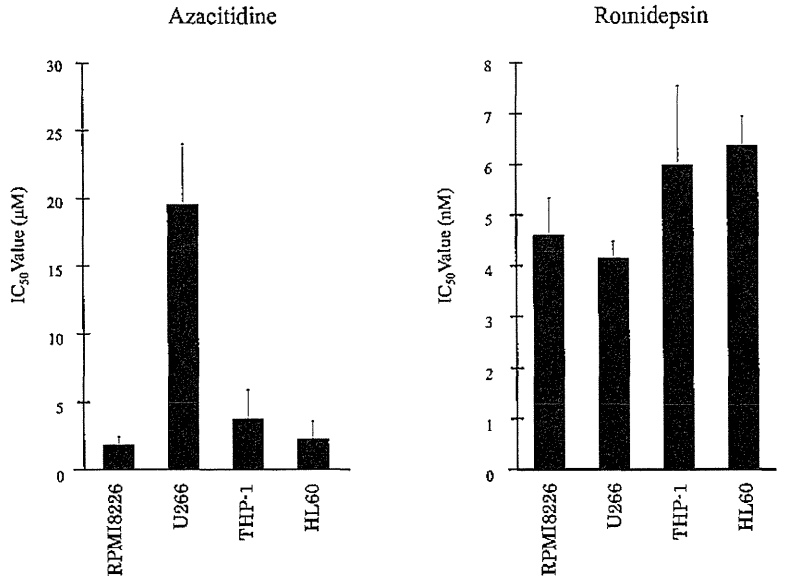


Figure 1. IC_{50} values of azacitidine and romidepsin against THP-1, HL60, RPMI8226 and U266 cells were determined as described in Materials and Methods. Experiments were repeated three times. Statistical analysis was carried out using Student's t-test for comparison of the data between THP-1 and each of other cell lines.

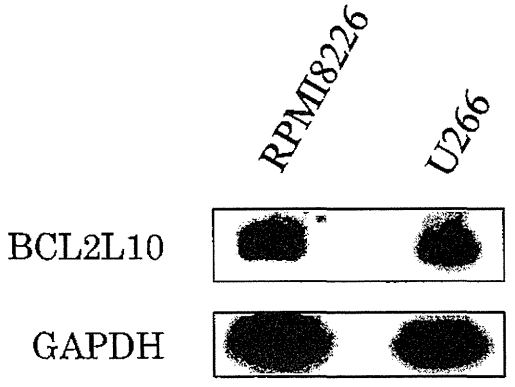


Figure 2. Total cell lysates were prepared and subjected to Western blot analysis using antibody against BCL2L10. The expression of GAPDH is shown as an internal control.

To reveal the mechanisms of resistance to azacitidine found in U266 cells, we first examined the levels of the anti-apoptotic factor BCL2L10 because a high expression level of this molecule was shown to be linked to azacitidine resistance in the leukemia cell line SKM1-R (Cluzeau T, *Oncotarget.* 2012). However, there was no remarkable difference in the protein levels between RPMI8226 and U266 cells (Figure 2).

DNMTs are the main target molecules of azacitidine. Thus, we next examined whether azacitidine-mediated disruption of DNMTs was abrogated. As shown in Figure 3A, azacitidine significantly reduced the levels of three DNMT isozymes, DNMT1, DNMT3a and DNMT3b in RPMI8226 cells, whereas it showed little suppressive effect on the levels of these enzymes in U266 cells. Consistent with these results, DNMT activity was inhibited with azacitidine treatment in RPMI8226 cells but not in U266 cells. (Figure 3B). These results suggest that loss of anti-MM effect of azacitidine in U266 cells was due to diminishment of its demethylating activity. Recently, in newly established azacitidine-resistant human leukemia cell lines THP-1/AR and HL60/AR, we found heterozygous point mutations in exons 4 and 5 of the *UCK2* gene, which encodes a key enzyme for the azacitidine activation process (unpublished data). We therefore examined whether U266 cells also have point mutations in *UCK2* ; however, none were found (data not shown).

Combined romidepsin and azacitidine enhanced induction of apoptosis in RPMI8226 cells but not in U266 cells.

Figure 3A

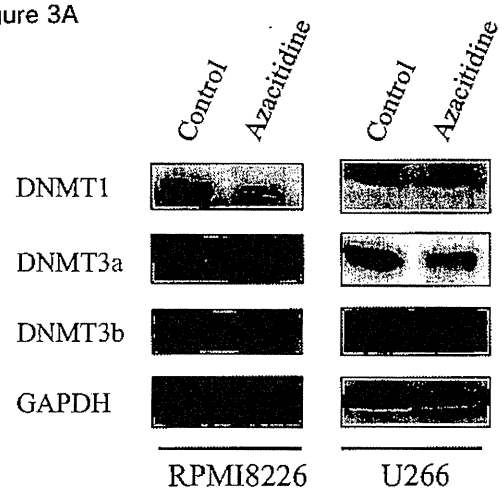


Figure 3B

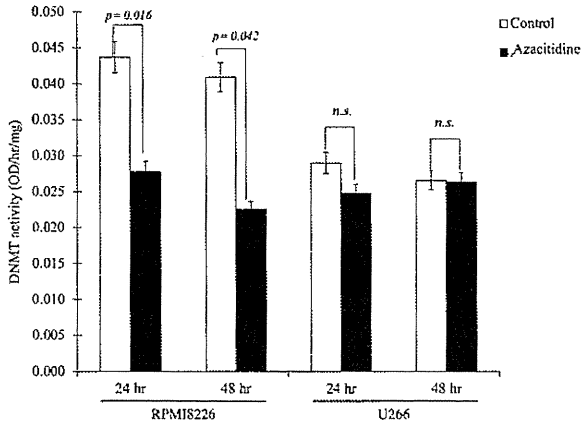


Figure 3. (A) Cells were treated with 4 μM azacitidine for 48 hours. Total cell lysates were prepared and subjected to Western blot analysis using antibody against DNMT1, DNMT3a and DNMT3b. The expression of GAPDH is shown as an internal control. (B) Cells were cultured in 4 μM azacitidine for 24 or 48 hours. DNMT activity was determined using DNMT activity/inhibition assay system (ActiveMotif, Carlsbad, CA). Statistical analysis was carried out using Student's t-test.

Figure 4A

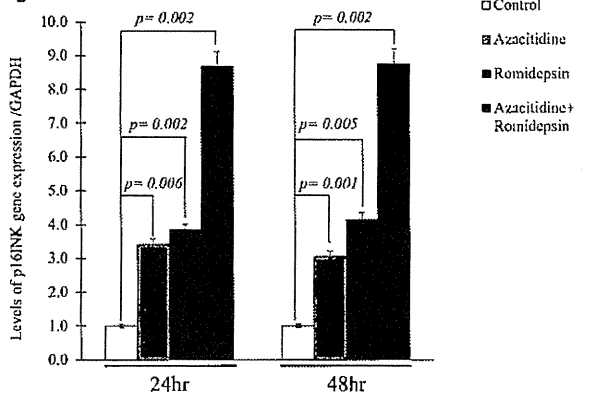


Figure 4B

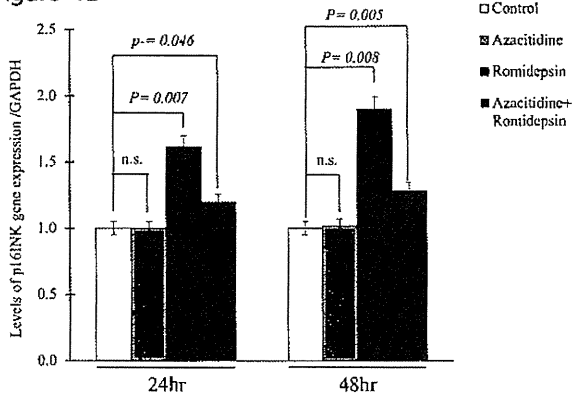


Figure 4C

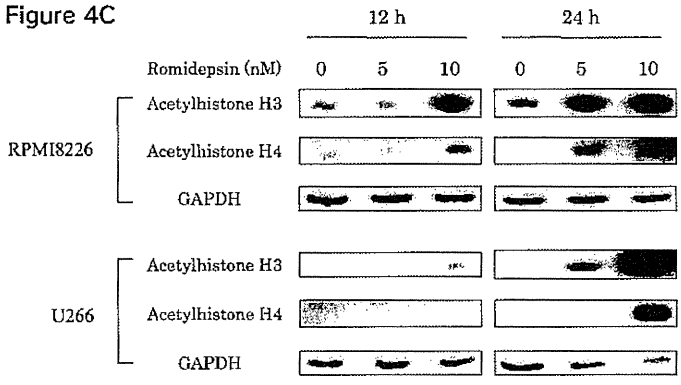


Figure 4. RPMI8226 cells (A) or U266 cells (B) were treated with 4 μM azacitidine alone, 10 nM romidepsin alone, or the combination of azacitidine and romidepsin for 24 or 48 hours. The levels of *p16* mRNA were determined by real-time PCR analyses as described in Materials and Methods. The results were calculated using the DDC_T method and are expressed as the ratio of *p16* mRNA level in cells treated with azacitidine or romidepsin to that in untreated cells. Statistical analysis was carried out using Student's t-test for comparison of the data between untreated cells and cells treated with each agent. (C) Cells were incubated with indicated concentrations of romidepsin for 12 or 24 hours. Total cell lysates were prepared and subjected to Western blot analysis using antibody against acetylhistone H3 and acetylhistone H4.