Original Article

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

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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),

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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.

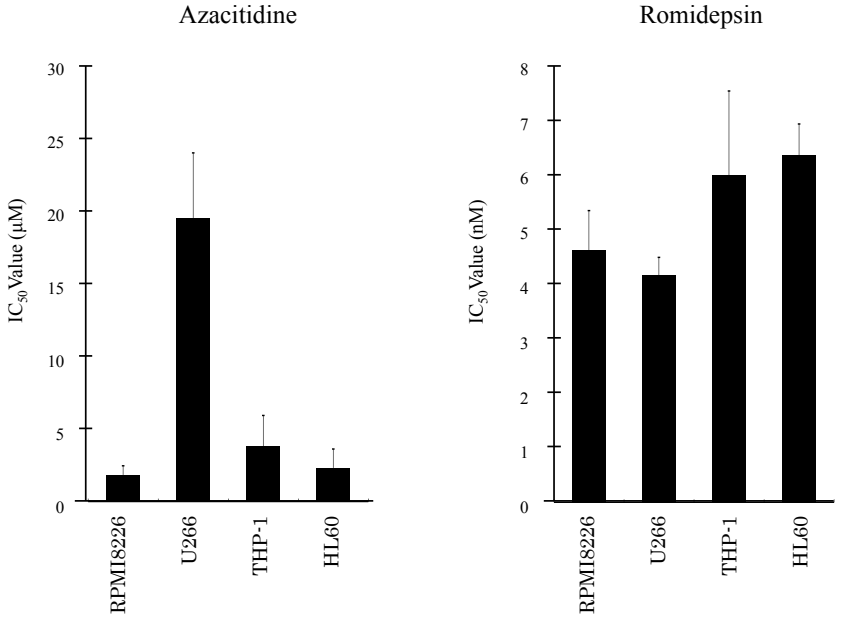

Figure 1. IC_{50} values of azacitidine and romidepsin against THP-1, HL60, RPMI8226 and U266 cells were determined as described in Materials and Methods. Experiments were repeated three times. Statistical analysis was carried out using Student's t-test for comparison of the data between THP-1 and each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

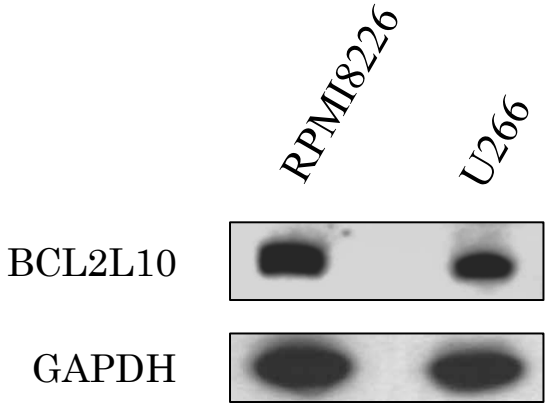

Figure 2. Total cell lysates were prepared and subjected to Western blot analysis using antibody against BCL2L10. The expression of GAPDH is shown as an internal control.

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

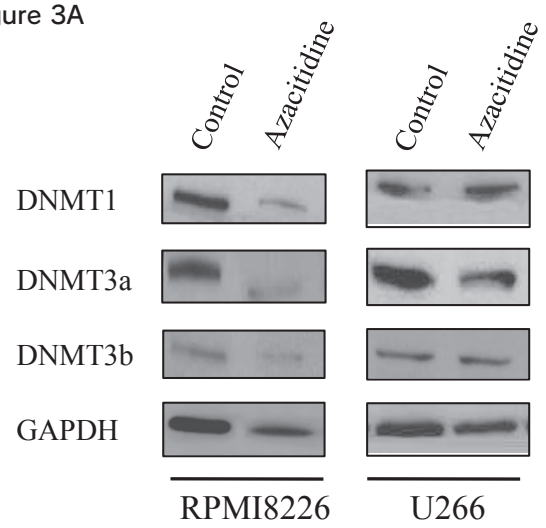

Figure 3B

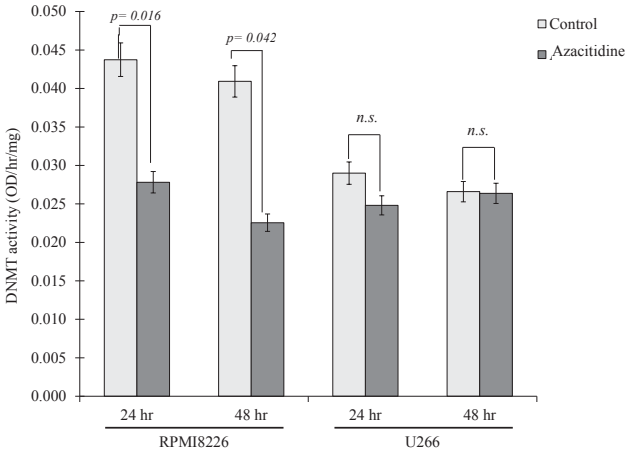

Figure 3. (A) Cells were treated with 4 μM azacitidine for 48 hours. Total cell lysates were prepared and subjected to Western blot analysis using antibody against DNMT1, DNMT3a and DNMT3b. The expression of GAPDH is shown as an internal control. (B) Cells were cultured in 4 μM azacitidine for 24 or 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

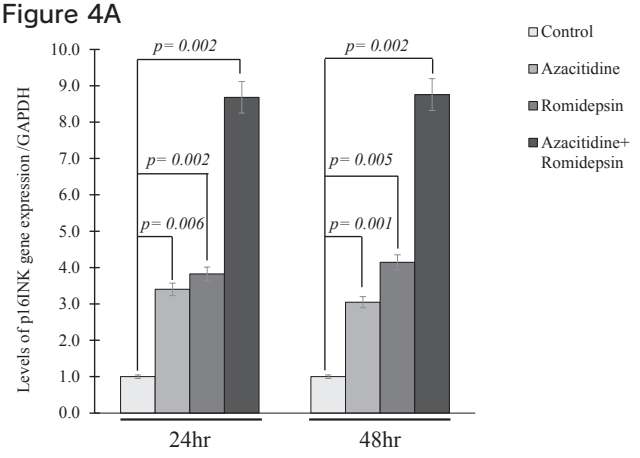

Figure 4B

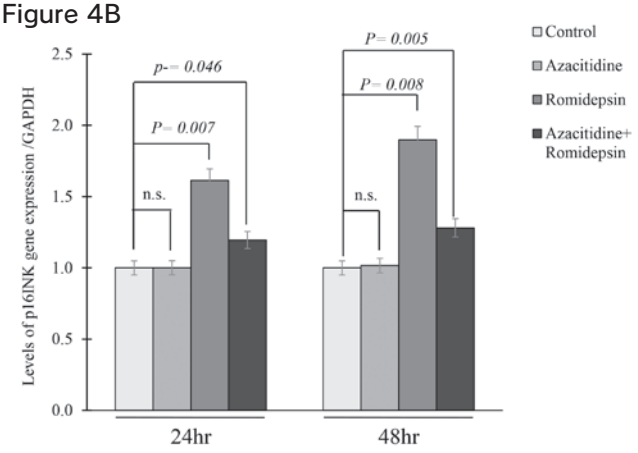

Figure 4C

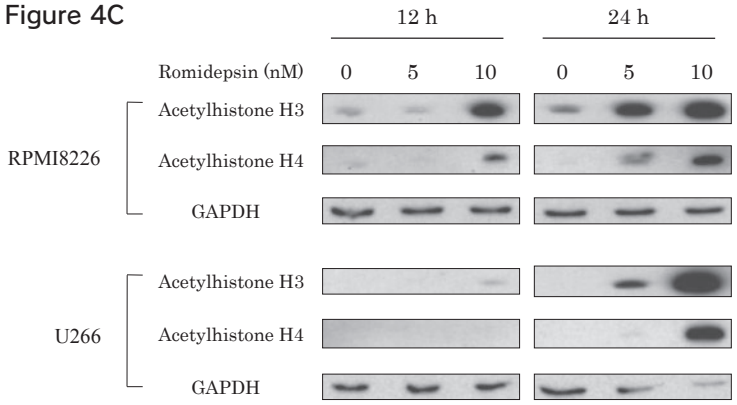

Figure 4. RPMI8226 cells (A) or U266 cells (B) were treated with 4 μM azacitidine alone, 10 nM romidepsin alone, or the combination of azacitidine and romidepsin for 24 or 48 hours. The levels of *p16* mRNA were determined by real-time PCR analyses as described in Materials and Methods. The results were 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.

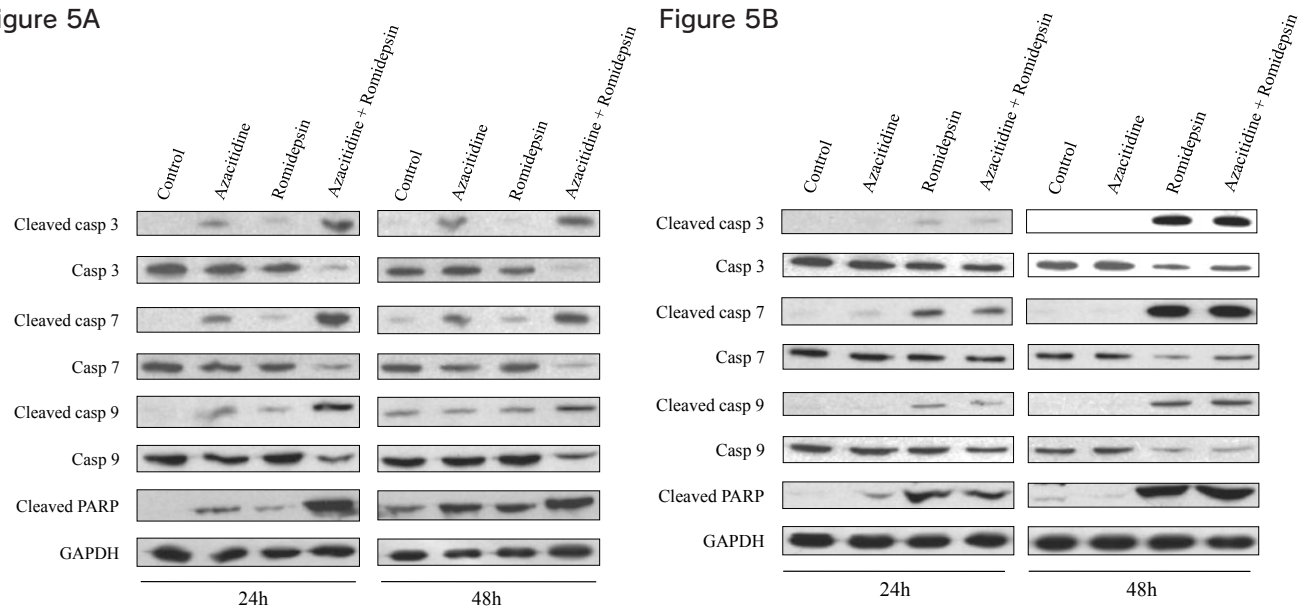

Figure 5. RPMI8226 cells (A) or U266 cells (B) were treated with 4 μM azacitidine alone, 10 nM romidepsin alone or the combination of azacitidine and romidepsin for 24 or 48 hours. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading.

According to changes in the level of DNMT activity, the mRNA level of the tumor-suppressor gene *p16* increased with azacitidine treatment in RPMI8226 cells, whereas in U266 cells, it remained constant (Figure 4A-B). On the other hand, romidepsin effectively increased the levels of acetylated H3 and acetylated H4 in both RPMI8226 and U266 cells (Figure 4C). Accordingly, romidepsin induced *p16* gene expression in both cell lines, suggesting that romidepsin-mediated histone acetylation promoted gene expression in MM cells (Figure 4A-B). Interestingly, the combination of azacitidine and romidepsin markedly enhanced the induction of *p16* mRNA level in RPMI8226 cells (Figure 4A). However, in U266 cells, there was no remarkable difference in the level of *p16* mRNA between cells treated with the combination and those with romidepsin alone (Figure 4B). These results raised the possibility that the combination enhances anti-tumor effect in RPMI8226 cells but not in U266 cells.

To verify this hypothesis, we examined the effects of azacitidine and romidepsin on the levels of apoptosis-related molecules. As expected, the treatment of RPMI8226 cells with azacitidine alone or romidepsin alone resulted in increased levels of apoptosis-related molecules including the cleaved form of caspase-3, caspase-7, caspase-9 and PARP (Figure 5A), whereas in U266 cells, only romidepsin increased the levels of these molecules (Figure 5B). Importantly, the combination of azacitidine and romidepsin significantly augmented the increase in the levels of all these cleaved proteins in RPMI8226 cells (Figure 5A). In contrast, in U266 cells, the combination had no stronger effect on the levels of these molecules than romidepsin alone (Figure 5B). Taken together, the results suggest that the combination effectively enhanced induction of apoptosis by activation of the caspase pathway in RPMI8226 cells but not in U266 cells.

Combined azacitidine and romidepsin additively inhibited the growth of RPMI8226 cells.

We finally examined the cytotoxic effects of the combination of azacitidine and romidepsin by Steel and Peckham isobologram analysis. The dose-response curves for azacitidine in combination with romidepsin are shown in Figure 6A. Isobolograms were then created on the basis of the results of the dose-response curves. As shown in Figure 6B, all points for the combination of azacitidine and romidepsin lie within the envelope formed by two broken lines in RPMI8226 cells, suggesting that the combination had an additive effect on inhibition of cell growth. In contrast, in U266 cells, some of the points lie outside the right margin of the envelope, suggesting that the combination showed a nearly antagonistic effect on inhibition. These results also suggest that romidepsin combined with azacitidine augments anti-tumor effect in azacitidine-sensitive RPMI8226 cells but not in azacitidine-resistant U266 cells.

Discussion

In RPMI8226 cells, azacitidine restored p16 gene expression accompanied by the disruption of DNMT, suggesting that azacitidine showed anti-tumor effects through alteration of epigenetic status. In contrast, it had no effect on U266 cells, in which azacitidine-mediated

Figure 6A

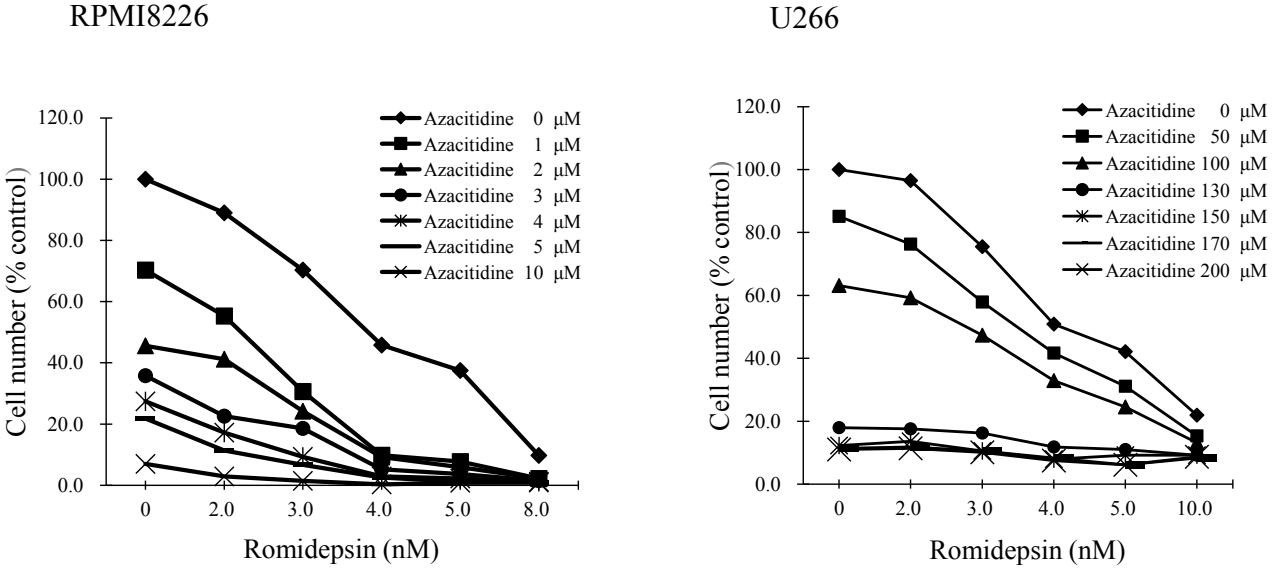

Figure 6B

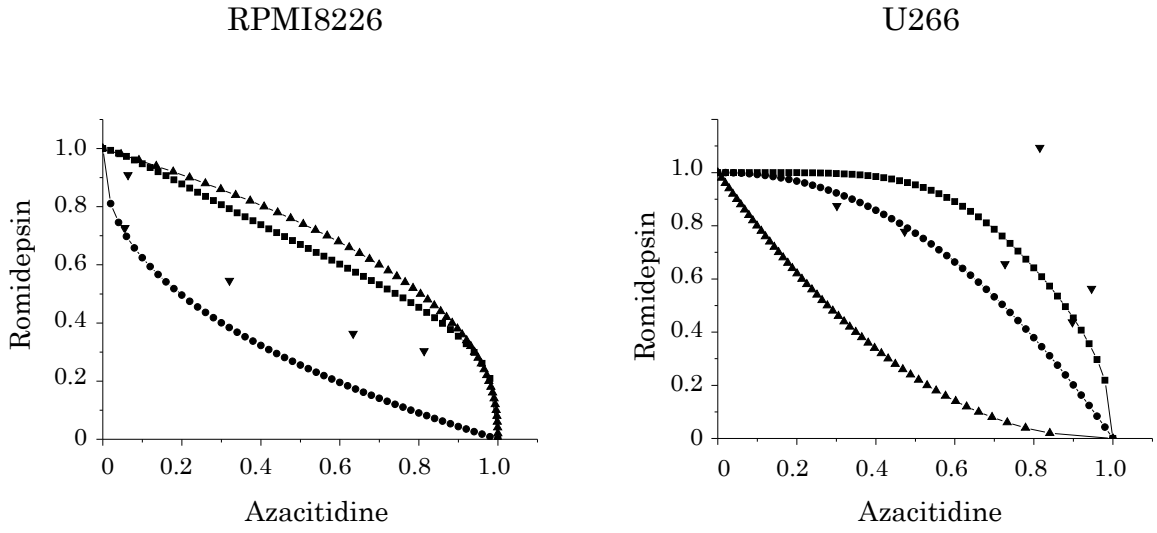

Figure 6. (A) Dose-response curves of azacitidine in combination with romidepsin were created. Cells were cultured with indicated concentrations of azacitidine for 96 hours in the presence of various concentrations of romidepsin, and the number of viable cells was counted with a Cell Counting Kit-8. (B) Steel and Peckham isobologram analysis of the combinations of azacitidine and romidepsin were performed as described in Materials and Methods. The concentrations that produced 50% cell growth inhibition were expressed as 1.0 on the ordinate and the abscissa of the isobolograms.

suppression of DNMT activity was negated (Figure 3B). The mechanisms underlying azacitidine resistance are poorly understood, so clarifying them is important. Cluzeau et al. recently reported that increased expression of the anti-apoptotic factor BCL2L10 was linked to drug resistance in their azacitidine-resistant leukemia cell line SKM1-R (Cluzeau T, *Oncotarget.* 2012). However, in U266 cells, BCL2L10 might not be involved in the acquisition of resistance because diminished azacitidine activation could be the essential factor. Indeed, the protein level of BCL2L10 in U266 cells compared with RPMI8226 cells was essentially unchanged (Figure 2).

Diminished azacitidine activation might be caused by perturbation of the azacitidine activation process, in which uridine-cytidine kinase 2 (UCK2) functions as a key enzyme. We recently cloned two azacitidine-resistant human leukemia cell lines and found that UCK2 gene mutations were fundamentally involved in their azacitidine-resistance (unpublished data). However, in U266 cells, neither UCK2 gene mutations nor a reduction of UCK2 protein level was found (data not shown). Nevertheless, activity of azacitidine in U266 cells diminished ; therefore, other mechanisms must be involved. One possibility is that the transport of azacitidine into cells is reduced, resulting in low concentrations of azacitidine in U266 cells. It has been shown that equilibrative nucleoside transporters and

concentrative nucleoside transporters play an important role for transport of natural nucleosides and nucleoside analogs into cells (Damaraju VL, Oncogene. 2003, Kong W, Curr Drug Metab. 2004, Pastor-Anglada M, Leukemia. 2004) ; therefore, decreasing levels of these transporters is a possible mechanism for acquiring resistance to azacitidine, a nucleoside analog of cytidine.

Romidepsin was effective on U266 cells as well as RPMI8226 cells. HDIs alter the acetylation status of not only histone proteins but also non-histone proteins (Bali P, J Biol Chem. 2005). However, the main targets of romidepsin are HDAC1, HDAC2 and HDAC6 (Klimek VM, Clin Cancer Res. 2008). Since HDAC1 and HDAC2 belong to class 1 HDACs, which alter epigenetic status through histone deacetylation, it is possible that romidepsin-mediated alteration of aberrant epigenetic states plays an important role in its anti-MM activity. The results of Steel and Peckham isobologram analyses, which provide very strict and reliable results for cytotoxic effects of combination treatments (Steel GG, *Int. J.Radiat.Oncol.Biol.Phys.* 1979), show that the combination of azacitidine and romidepsin had an additive effect on suppressing growth of RPMI8226 cells, whereas it had a nearly antagonistic effect in U266 cells. In RPMI8226 cells, the combination may augment the effect on epigenetic status, resulting in enhancement of cytotoxic effects. In fact, the level of p16 mRNA markedly increased with the combination in RPMI8226 cells (Figure 4A). These results thus suggest that combined azacitidine and romidepsin is effective only in azacitidine-sensitive MM cells.

In conclusion, some MM cells might be resistant to azacitidine. The combination of azacitidine and romidepsin is a potential therapy for MM ; however, this combination might not be effective for azacitidine-resistant MM cells.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

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アザシチジン感受性および耐性多発性骨髄腫細胞株に対するアザシチジンとロミデプシンの併用効果

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要　　約

造血器腫瘍においては，DNAの高メチル化やヒストン蛋白の脱アセチル化などが高頻度に観察されており，脱メチル化薬やヒストン脱アセチル化酵素阻害薬（以下HDI）が期待されている。本研究では，ヒト多発性骨髄腫（以下MM）細胞株RPMI8226とU266を用いて脱メチル化薬であるazacitidineとHDIであるromidepsinの効果を検討した。RPMI8226では，azacitidineによるDNAメチル転移化酵素活性の抑制およびp16 mRNA発現誘導を認め，その結果，アポトーシスが誘導された。しかしながら，U266ではazacitidineによるDNAメチル転移化酵素阻害作用が機能せず，抗腫瘍効果も認めなかった。一方，romidepsinによるアポトーシス誘導は両細胞において認められた。RPMI8226では両薬剤の併用によりp16 mRNA とcleaved formのcaspases量の著明な増加を認めたが，U266では認めなかった。さらに，isobologram解析では両薬剤による相加的抗腫瘍効果をRPMI8226で認めたが，U266では拮抗作用に近い結果であった。以上の結果は，MMに対するazacitidineとromidepsinの併用療法がazacitidien感受性細胞にのみ有効であることを示唆している。

（キーワード：アザシチジン，DNA脱メチル化薬，ヒストン脱アセチル化酵素阻害薬，多発性骨髄腫，ロミデプシン）

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