Mechanisms of resistance to azacitidine in human leukemia cell lines

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Abstract

The DNA methylation inhibitor azacitidine (5-azacytidine) is used against myelodysplastic syndrome and acute myeloid leukemia, but drug resistance is an ongoing, intractable problem. To investigate resistance mechanisms, we generated two azacitidine-resistant cell lines, THP-1/AR and HL60/AR, and studied genetic disparities between them and their corresponding parental lines. In cells treated with azacitidine, significant mitotic variations were noted in parental cells which were absent in resistant cells, suggesting that resistance arises from negating azacitidine-mediated activation of apoptosis signaling and reestablishing G_2/M checkpoint. Importantly, both resistant cell lines have common point mutations in the uridine-cytidine kinase 2 (*UCK2*) gene, which encodes the rate-limiting enzyme of the azacitidine activation pathway. Forced expression of mutated *UCK2* in parental THP-1 cells abrogated azacitidine-induced apoptosis, whereas over-expression of wild-type *UCK2* in resistant THP-1/AR cells restored sensitivity to azacitidine, implying that *UCK2* gene mutations perturb azacitidine activation and advance azacitidine resistance. Our study provides new insights into azacitidine resistance and establishes models useful in developing effective strategies to overcome it.

Key Words: azacitidine, drug resistance, acute myeloid leukemia, uridine-cytidine kinase, UCK2

In leukemia cells, the methylation states of gene regulatory regions are up-regulated, thereby silencing the expression of some genes, including tumor suppressor genes and cell-differentiation related genes [1-6]. Abrogation of hypermethylation should promote re-expression of these genes, consequently inducing anti-tumor effects. For this purpose, two DNA methyltransferase (DNMT) inhibitors azacitidine (5-azacytidine) and decitabine (5-aza-2-deoxycytidine) were developed. Although both are nucleoside analogs of cytidine, their anti-tumor mechanisms differ. Decitabine incorporates into DNA, resulting in disruption of DNMT, thus suppressing DNA methylation. Azacitidine, likewise, can incorporate into DNA, suppressing methylation; however, it preferentially incorporates into RNA, developing cytotoxicity by inhibiting protein synthesis [7-8]. The anti-tumor effects of azacitidine are concentration-dependent. Its cytotoxic activity increases with concentration; whereas, its DNMT inhibition peaks at low concentrations and abates at higher concentrations [7-10]. Although their anti-tumor mechanisms differ, in preclinical examinations and clinical trials both reagents have been shown to possess cytotoxic properties and differentiation-inducing effects [11-15]. Accordingly, both are now approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia (AML) with 20% to 30% bone marrow blasts, and their usefulness for treating AML with high bone marrow blasts is under investigation [16].

Drug resistance remains a major problem for patients treated with small molecular reagents such as azacitidine, but the underlying mechanisms are poorly understood [17]. Therefore, clarifying resistance mechanisms is central to develop effective leukemia therapies. Previous studies have shown that human equilibrative nucleoside transporters (hENTs) and concentrative nucleoside transporters (hCNTs) play an important role for transport of natural nucleosides and nucleoside analogs into cells [18-20]. Using hCNT1-expresing kidney cells, Rius et al. showed that hCNT1 mediates uptake of azacitidine, thus sensitizing cells [21]. This suggests that decreasing levels of these transporters is a possible mechanism for acquiring resistance to DNMT inhibitors. However, both azacitidine and decitabine undergo a series of phosphorylation steps before incorporation into DNA or RNA. In this

process, uridine-cytidine kinase 2 (UCK2) and deoxycytidine kinase (DCK) function as key enzymes for activation of azacitidine and decitabine respectively. Therefore, perturbation of the activation process may also result in blocking nucleoside analog activity, even with normal uptake [22]. Indeed, Qin T et al. found that the level of DCK attenuated due to a *DCK* gene mutation in decitabine-resistant leukemia cells [23]. Other scenarios postulate that DNMT inhibitors are certainly active in leukemia cells but fail to kill them, in which case, resistance mechanisms probably involve continuous activation of cellular signaling pathways such as the Ras-ERK pathway, or deactivation of apoptotic pathways. In fact, Cluzeau et al. recently reported that increased expression of the anti-apoptotic factor BCL2L10 was linked to resistance in azacitidine-resistant cell line SKM1-R [24]; but this mechanism is not well elucidated. However, a recent study showed that cytidine deaminase inactivates DNMT inhibitors, decreasing their half-life [25]. Therefore, various mechanisms, either common or specific to each reagent, may be involved in acquisition of resistance to DNMT inhibitors; but most remain obscure.

In this study, we developed two azacitidine-resistant cell lines to probe the processes involved in azacitidine drug resistance. Our results demonstrate that acquisition of resistance is caused by perturbation of the azacitidine activation process due to *UCK2* gene mutations.

Materials and Methods

Reagents

Azacitidine, decitabine and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines

THP-1 and HL60 are BCR/ABL-negative human myeloid leukemia cell lines [26-27]. To determine the IC_{50} values of azacitidine, cells from the two parental

lines were independently 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. Dose response curves were prepared, and concentrations yielding 50% cellular viability were designated as IC_{50}.

To generate resistant clones, THP-1 and HL60 cells were treated with step-wise increasing concentrations of azacitidine (0.2 μM to 1.0 μM) and colonized on a medium containing methylcellulose with 1.0 μM azacitidine, followed by selection and cloning of surviving colonies. Clones at the highest IC_{50} value from each cell line were designated as THP-1/AR (TAR) and HL60/AR (HAR). All cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin G, and streptomycin sulfate; and split every 4 days.

Flow cytometry

For cell cycle analysis, cells were incubated with propidium iodide for 15 minutes, and then analyzed by flow cytometry using a FACScan/CellFIT system (Becton Dickinson, San Jose, CA). To analyze p-glycoprotein expression, cells were incubated with a phycoerythrin-labeled anti-p-glycoprotein antibody for 30 minutes. Phycoerythrin-labeled mouse IgG1 was used as a control. To examine the expression levels of surface antigens including CD11b, CD13, CD14, CD15, CD33, CD36 and HLA-DR, cells were incubated in solutions of respective antibodies conjugated with fluorescein isothiocyanate (Becton Dickinson, San Jose, CA) for 30 minutes, then analyzed by flow cytometry.

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 [28]. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, used as an internal control, was purchased from Chemicon International

(Temecula, CA). Anti-caspase-3, anti-cleaved caspase-3, anti-caspase-7, anti-cleaved caspase-7, anti-caspase-9, anti-cleaved caspase-9, anti-p44/42 (ERK1/2) MAP kinase, anti-phospho p44/42 (ERK1/2) MAP kinase, anti-JNK/SAPK, and anti-phospho JNK/SAPK were purchased from Cell Signaling Technology (Beverly, MA). Anti-DNA methyltransferase 1 (DNMT1), anti-DNMT3a and anti-DNMT3b were purchased from ActiveMotif (Carlsbad, CA). Anti-uridine-cytidine kinase 2 (UCK2) rabbit polyclonal antibody was purchased from Proteintech Group, Inc. (Chicago, IL). Anti-BCL2L10 antibody was purchased from Cell Signaling Technology (Beverly, MA).

DNMT activity assay

Cells were cultured in the presence or absence of azacitidine for 24 hours. Nuclear extracts were then prepared as described previously [29]. DNMT activity was determined using a DNMT activity/inhibition assay system (ActiveMotif, Carlsbad, CA) in accordance with the manufacturer's instructions.

Sequence analysis

Total RNA from cells was isolated by the acid guanidium thiocyanate phenol chloroform method. Polymerase chain reaction (PCR) was performed using cDNA that had been prepared from total RNA by Superscript II reverse transcriptase. The primers used for cDNA amplification are summarized in Supplementary Table 1. Direct sequence analysis was performed using primers summarized in Supplementary Table 2.

Real-time PCR analysis

We generated cDNA from total RNA extracted by reverse transcriptase and subjected to SYBR real-time PCR quantitation. 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 for real-time PCR are summarized in

Supplementary Table 3. The results were calculated using the DDC_T method.

Transfection of expression vectors

Full length of wild type *UCK2* cDNA (NCBI Reference Sequence: NM_012474.4) and *UCK2* cDNA with five point mutations, which were found in TAR cells, were amplified by PCR using cDNA generated from total RNA that had been extracted from THP-1 and TAR cells, respectively. PCR fragments were subcloned into PMD20-T vector (Takara Bio Inc., Shiga, Japan). Expression vectors of wild type and mutated *UCK2* were constructed by insertion of wild type *UCK2* cDNA and mutated *UCK2* cDNA into the Hpa I and Xho I site of the pLL3.7 plasmid, which yielded UCK2wt and UCK2mut. Transfection of these vectors into THP-1 or TAR cells was carried out using X-tremeGENE transfection reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. Briefly, 3 μg of the expression vector and 9 μL of X-tremeGENE HP DNA Transfection Reagent were mixed in 100 μL of Opti-MEM1 medium without serum and incubated for 30 minutes. Then the mixture was added to 1 X 10^6 cells in 500 μL of fresh Opti-MEMI medium and further incubated for 48 hours. To isolate cells that were successfully transfected with the vectors, GFP-positive cells were collected using FACSAriaTM□Cell Sorter (Beckton Dickinson Co., Ltd.).

Transfection of small interfering RNA (siRNA)

One μg of BCL2L10 or control random siRNAs were transfected using XtremeGENE siRNA transfection reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. BCL2L10 and control random siRNAs were purchased from Qiagen Inc. (Valencia, CA).

Results

Abrogation of apoptosis and G_2/M arrest

The IC_{50} values of azacitidine against our drug-resistant clones were markedly higher than those against the corresponding parental cell lines, indicating that the clones had acquired significant resistance (Fig. 1A). Flow cytometry analysis showed that both azacitidine-resistant and their corresponding azacitidine-sensitive cells have the same expression pattern of surface antigens including CD11b, CD13, CD14, CD15, CD33, CD36 and HLA-DR (data not shown). Furthermore, there were no differences in the levels of proliferation abilities between azacitidine-resistant and azacitidine-sensitive cells (Fig. 1B). However, the levels of differentiation mediated by phorbol 12-myristate 13-acetate (PMA) were lower in azacitidine-resistant cells than in azacitidine-sensitive cells (Fig. 1C-D). Therefore, it remains possible that the differentiation process was disturbed in azacitidine-resistant clones.

To explore the molecular mechanisms behind this resistance, we examined regulation of the cell cycle by flow cytometry analysis (Fig. 2A). In the sensitive cell lines, azacitidine increased the percentage of sub-G_1 and G_2/M-phase cells and decreased S-phase cells. However, in the resistant lines, it had no similar effect, suggesting that azacitidine-mediated activation of both the G_2/M checkpoint and apoptotic activity was abrogated.

To confirm abrogation of apoptosis signaling, we examined levels of apoptosis-related molecules. Consistent with the results of flow cytometry analysis, the levels of cleaved caspase-3, cleaved caspase-7, and cleaved caspase-9 increased with azacitidine treatment in sensitive cells in a dose-dependent manner; whereas in resistant cells, these levels remained constant (Fig. 2B). We also examined whether the effect of azacitidine on JNK/SAPK signaling differed between sensitive and resistant cells because it is well known that induction of G_2/M arrest is associated with JNK/SAPK activation [30-33]. Azacitidine markedly elevated the level of phospho JNK/SAPK in sensitive cells but not in resistant cells (Fig. 2C). In contrast, the level of phospho ERK1/2, in the MAP kinase subfamily, increased with azacitidine treatment in both sensitive and resistant cells.

DMNT activity in resistant cells

Two mechanisms can explain azacitidine resistance: diminished activity and failed cytotoxicity. In the latter case, continuous activation of alternative signaling pathways is probably involved. To clarify whether resistance relates to diminished activity, we examined azacitidine's effect on DNMTs, its main target molecules, particularly in low concentrations. We found that it inhibited DNMT activity in sensitive cells, but not in resistant cells (Fig. 3A). To confirm this, we performed Western blot analysis using anti-DNMT antibodies. Consistent with the results of the DNMT activity assay, Western blot showed that azacitidine significantly reduced levels of three DNMT isozymes, DNMT1, DNMT3a, and DNMT3b in sensitive cells; but not in resistant cells (Fig. 3B). Furthermore, changes in the levels of DNMT activity accompanied expression of the tumor-suppressor gene *p16* in sensitive cells, but not in resistant cells (Fig. 3C). We also confirmed by sequence analysis that there was no mutation of the DNMT genes in resistant cell lines (data not shown). These findings thus suggest that diminished activity is central to azacitidine resistance.

Expression of membrane transporters

To reveal the mechanisms underlying the diminished azacitidine activity in resistant cells, we first examined the levels of membrane transporters that may play a role in transporting azacitidine into cells. The results of real-time PCR showed that the expression level of equilibrative nucleoside transporter hENT1 was lower in TAR cells than those in THP-1 cells (Fig. 4A); however, the difference was unremarkable. There was no significant difference in the expression levels of another equilibrative nucleoside transporter hENT2 nor the concentrative nucleoside transporter hCNT1. Additionally, the levels of p-glycoprotein, a member of the ABC transporters, were not elevated in either resistant cell line (Fig. 4B). These results suggest that alteration of azacitidine transport is not meaningfully involved in azacitidine-resistance.

UCK2 gene mutations in resistant cells

We then hypothesized that the diminished azacitidine activity is caused by perturbation of the azacitidine activation process. To verify this hypothesis, we examined the protein levels of uridine-cytidine kinase 2 (UCK2), a key azacitidine activation enzyme, by Western blot analysis; however, there was no remarkable difference in the levels of UCK2 between resistant cells and their corresponding sensitive cells (Fig. 5A). However, in exons 4 and 5 of the *UCK2* gene we found four heterozygous point mutations in HAR cells and five in TAR cells, of which four were identical to the HAR mutations (Fig. 5B). To clarify whether these gene mutations play a role in azacitidine resistance, we examined the effect of forced expression of *UCK2* on azacitidine sensitivity. As shown in Fig. 5C, over-expression of mutated *UCK2* in THP-1 cells slightly increased the IC_{50} value of azacitidine; but without statistical significance. However, azacitidine-mediated induction of cleaved caspases as well as p16 expression was abrogated (Fig. 5D-E). Furthermore, transfection of the wild type *UCK2* expression vector into TAR cells resulted in a significant decrease in the IC_{50} value of azacitidine (Fig. 5C) as well as restoration of azacitidine-induced apoptosis signaling (Fig. 5D). Since azacitidine-mediated induction of p16 expression was restored in the wild type *UCK2*-transfected TAR cells (Fig. 5E), it is highly possible that the azacitidine activation pathway was reestablished in these cells. These results thus suggest that the *UCK2* gene mutations found in resistant cells are critically involved in the resistance mechanism. It is likely that in resistant cells these gene mutations reduced UCK2 activity, resulting in perturbation of azacitidine activation and the consequent failure to suppress DNMT.

BCL2L10 expression in resistant cells

We finally examined the role of BCL2L10, which is a member of the anti-apoptotic BCL2 family and has been shown to be linked to drug resistance in another azacitidine-resistant cell line SKM1-R [24]. Interestingly, BCL2L10 was substantially expressed in both TAR and HAR cells, whereas the protein level of this molecule was extremely low in the parental cells (Fig. 6A). However, siRNA-mediated knockdown of BCL2L10 resulted in no restoration of

azacitidine-sensitivity in resistant cells (Fig. 6A-B) probably because azacitidine could not be activated regardless of the level of BCL2L10.

Discussion

Although clarification of mechanisms of resistance to DNA methyltransferase (DNMT) inhibitors is very important for clinical application, only a few resistant cell lines have been reported [23, 34]. Our new cell lines are thus opportune models for analyzing mechanisms of resistance to azacitidine in leukemia cells.

Azacitidine shows anti-tumor effect through two major mechanisms, namely, disruption of DNA methylation due to DNMT inhibition, and induction of cytotoxicity due to inhibition of protein synthesis [7-8]. The proportion of each mechanism depends on concentration; low concentrations favoring the former and high concentrations the latter. In patients it has been reported that maximal blood concentration (Cmax) of azacitidine is low (750.0 ± 403.3 ng/mL (equivalent to 3.07 ± 1.65 μM) by subcutaneous injection) [35], suggesting that DNMT inhibition is the dominant mechanism in clinical applications. Therefore, we examined the effect of low concentrations of azacitidine (2μM and 4μM) in this study. Although the detailed mechanisms regarding the effects on cell functions of suppressing DNA methylation remain unclear, it has been found that a low concentration of azacitidine leads to apoptosis as well as cell differentiation [8].

Consistent with previous reports, our results show that azacitidine induces apoptosis signaling in THP-1 and HL60 cells. Since this was not observed in our resistant cell lines, abrogation of these effects is clearly involved in acquisition of azacitidine-resistance. Interestingly, azacitidine also induced accumulation of G_2/M-phase cells with a concomitant decrease in S-phase cells in parental cell lines (Fig. 2A), which was consistent with the previous findings that azacitidine induces cell cycle blockage in the G_2/M-phase [12]. Furthermore, it significantly increased the levels of phospho-JNK. It has been reported that activation of JNK/SAPK signaling is

involved in cell-cycle blockage at G_2/M phase in some cells [30-33]. Since this activation effect was not observed in resistant cells (Fig. 2C), it is possible that azacitidine activates the G_2/M checkpoint through activation of JNK/SAPK signaling, and that interrupting induction of cell-cycle blockage bears upon the acquisition of resistance.

Since both THP-1 and HL60 cells primarily expressed sufficient levels of granulocyte-specific antigens such as CD33 and CD13, we could not establish that azacitidine mediated induction of myeloid differentiation. Furthermore, azacitidine had no effect on cell morphology or on the levels of monocyte-macrophage specific antigens including CD11b, CD14, CD36 and HLA-DR in azacitidine-resistant cells as well as in azacitidine-sensitive cells (data not shown). Therefore, we could not determine whether azacitidine-mediated differentiation is also abrogated in azacitidine-resistant cells.

In both resistant lines, the inhibitory effect of azacitidine on DNMT activity was completely abrogated, meaning that resistance was acquired by a DNMT-dependent mechanism. A failure of the azacitidine activation process is one possible mechanism for DNMT-dependent resistance. In this study, we found some point mutations in exons 4 and 5 of the *UCK2* gene, which correspond to the kinase domain (Fig. 5B). Although direct evaluation of the enzymatic activity of mutated UCK2 and the concentration of the phosphorylated active form of azacitidine in cells was not performed in this study, it is probable that these mutations diminish UCK2 activity, resulting in perturbation of the azacitidine activation process. This hypothesis was supported by the observation that forced expression of wild type *UCK2* in the resistant cells restored sensitivity to azacitidine (Fig. 5C). Previously, Murata D et al. found a partial deletion in exon 5 of *UCK2* in a human fibrosarcoma cell line that was resistant to potent inhibitors of RNA polymerases 3'-ethynyl nucleosides [36]. They also found a point mutation in exon 4 of *UCK2* in the 3'-ethynyl nucleosides-resistant human gastric carcinoma cell line [36]. Therefore, it is highly possible that diminishment of UCK2 activity is critical for acquisition of

resistance to other anti-cancer drugs as well. In the previously reported decitabine-resistant cells, the level of DCK decreased due to a *DCK* gene mutation, resulting in the acquisition of resistance to decitabine as well as cytarabine, a key drug for leukemia treatment [23, 36]. In TAR and HAR cells, however, the level of UCK2 protein did not decrease, suggesting that those mutations diminish UCK activity without affecting the expression level of this enzyme.

Cluzeau T et al. showed that high expression of BCL2L10 was linked to drug resistance in their azacitidine-resistant cell line SKM1-R [24]. The azacitidine activation process might have been intact in SKM1-R cells because they showed that siRNA-mediated suppression of BCL2L10 restored azacitidine sensitivity. In our study we found that the protein level of BCL2L10 increased in both TAR and HAR cells (Fig. 6A). However, in our resistant cells, siRNA-mediated knockdown of BCL2L10 resulted in no restoration of sensitivity to azacitidine (Fig. 6B). These phenomena are consistent with our conclusion that azacitidine could not be activated due to *UCK2* gene mutations regardless of the level of BCL2L10.

It is of great importance to clarify whether *UCK2* gene mutations are present in primary cells from azacitidine-resistant patients and are central to the resistance mechanism. Although we have not yet analyzed sufficient numbers of patients, such research is now proceeding in our laboratory. It is also of interest to clarify whether other resistance mechanisms are also observed in primary cells.

Conclusion

Our newly established azacitidine-resistant cell lines THP-1/AR and HL60/AR are opportune models to analyze the mechanisms of azacitidine resistance. Using these cell lines, we revealed that acquisition of resistance is primarily caused by a DNMT-dependent mechanism due to *UCK2* gene mutations. These cell lines might also be useful to search for agents, which could overcome resistance to azacitidine.

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Conflict-of-interest disclosure

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Figure Legends

Figure 1. **Growth and phorbol 12-myristate 13-acetate (PMA)-mediated differentiation in azacitidine-resistant cells.** (A) IC_{50} values of azacitidine against THP-1, TAR, HL60 and HAR cells. Cells were first seeded at a density of 5×10^4 cells/mL and incubated in triplicate in the presence of various concentrations of azacitidine. Cell numbers were counted after 96 hours as described in Materials and Methods. Representative results for the dose-response growth inhibition curve are shown in the figure. Experiments were repeated three times. IC_{50} values of the cell lines are also shown in the figure. (B) Cells were incubated and harvested at various time points as indicated. The number of viable cells was counted at each time point using trypan blue staining. Experiments were repeated three times. Statistical analysis was carried out using Student's t-test for comparison of the data between azacitidine-resistant cells and their corresponding parental cells; but there were no differences at every point with statistical significance. (C) Cells were cultured in the absence or presence of PMA (50ng/mL) for 48 hours. Phase contrast microphotographs showed that THP-1 cells were changed to macrophage morphology while no morphological change was observed in TAR cells. PMA also failed to introduce HAR cells into adherent states. (D) Cells were cultured in the absence or presence of PMA (50ng/mL) for 48 hours. The positivity of surface antigens was determined by flow cytometry as described in Materials and Methods. The representative results of CD11b positivity were shown.

Figure 2. **Azacitidine-mediated induction of apoptosis and cell cycle blockage at G_2/M phase are abrogated in azacitidine-resistant cell lines.** (A) After azacitidine-resistant or corresponding parental cells had been incubated with indicated concentrations of azacitidine for 24 hours, the cells were harvested and incubated with propidium iodide for 30 minutes and analyzed by flow cytometry with a FACScan/CellFIT system (Becton Dickinson, San Jose, CA). Although addition of azacitidine resulted in an increase in the percentage of cells at G_2/M-phase and sub-G_1-phase in THP-1 and HL60 cells, these changes were not observed in TAR and