

Azacytidine Inhibits RNA Methylation at DNMT2 Target Sites in Human Cancer Cell Lines

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Abstract

The cytosine analogues azacytidine and decitabine are currently being developed as drugs for epigenetic cancer therapy. Although various studies have shown that both drugs are effective in inhibiting DNA methylation, it has also become clear that their mode of action is not limited to DNA demethylation. Because azacytidine is a ribonucleoside, the primary target of this drug may be cellular RNA rather than DNA. We have now analyzed the possibility that azacytidine inhibits the RNA methyltransferase DNMT2. We found that DNMT2 is variably expressed in human cancer cell lines. RNA bisulfite sequencing showed that azacytidine, but not decitabine, inhibits cytosine 38 methylation of tRNA^{Asp}, a major substrate of DNMT2. Azacytidine caused a substantially stronger effect than decitabine on the metabolic rate of all the cancer cell lines tested, consistent with an effect of this drug on RNA metabolism. Of note, drug-induced loss of RNA methylation seemed specific for DNMT2 target sites because we did not observe any significant demethylation at sites known to be methylated by other RNA methyltransferases. Our results uncover a novel and quantifiable drug activity of azacytidine and raise the possibility that tRNA hypomethylation might contribute to patient responses. [Cancer Res 2009;69(20):8127–32]

Introduction

The pharmacologic inhibition of DNA methylation plays an important role in the treatment of human cancers (1, 2). 5-Azacytidine (azacytidine) and the closely related compound 2'-deoxy-5-azacytidine (decitabine) are cytosine analogues that have been established as potent inhibitors of DNA methylation, both in preclinical models and in cancer patients (3). Both drugs have shown significant clinical benefits in a variety of clinical trials (4, 5) and have received Food and Drug Administration approval for the treatment of myelodysplastic syndrome, a preleukemic bone marrow disorder. It has also been shown in various studies that azacytidine and decitabine induce DNA demethylation in myelodysplastic syndrome patients (6–9), but DNA methylation biomarkers for the prediction of clinical patient responses remain to be established (3).

The human genome contains four DNA methyltransferase genes (10). DNMT1 is the major biochemical activity in protein extracts from various tissues and has been designated as the maintenance methyltransferase for the postreplicative maintenance of DNA methylation patterns. DNMT3A and DNMT3B are two closely related enzymes that have been termed *de novo* methyltransferases

and that are generally considered to play a major role in the establishment of methylation patterns. It has been shown that DNMT1, DNMT3A, and DNMT3B can be effectively depleted by azanucleosides (11). The mechanism of inhibition is widely assumed to depend on the incorporation of the fraudulent azacytosine base into DNA, followed by covalent trapping and subsequent degradation of the DNA methyltransferase enzymes. As a consequence, DNA will become passively demethylated when cells continue to replicate in the presence of reduced levels of DNA methyltransferase.

Despite the progress that has been made in characterizing the DNA demethylation responses of azacytidine and decitabine, numerous studies have indicated that the mode of action of these drugs is not limited to DNA methylation inhibition (3). In this context, it is particularly noteworthy that the effects of the ribonucleoside azacytidine on the RNA methyltransferase activity of the DNMT2 enzyme have not yet been investigated. DNMT2 contains all the catalytic signature motifs of conventional (cytosine-5) DNA methyltransferases, but has comparably low DNA methyltransferase activity (12). More recently, it was shown that DNMT2 methylates tRNA^{Asp} (13), which suggested that the substrate specificity of the enzyme might be different from other DNMTs. We have now analyzed the molecular effects of azacytidine in human cancer cell lines and found that the drug inhibits RNA methylation at DNMT2 target sites.

Materials and Methods

Cell culture. HCT116 cells were cultured in McCoy's 5a medium supplemented with 10% FCS. HL-60, K562, and ML-1 cells were cultured in RPMI 1640 supplemented with 5% L-glutamine, 10% FCS, 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Cells were treated with azacytidine (Sigma) or decitabine (Calbiochem), as indicated.

Dnmt2 expression analysis. RNA was extracted using the RNeasy RNA Isolation kit (Qiagen) and on-column DNA digestion was performed using the RNase-Free DNase Set (Qiagen). For quantitative reverse transcription-PCR (RT-PCR), 5 µg of RNA were reverse transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Each cDNA sample was analyzed in triplicate using the QuantiFast SYBR Green PCR kit (Qiagen). QuantiTect Primer Assays (Qiagen) were used for DNMT1 (Hs_DNMT1_1_SG, QT00034335) and DNMT2 (Hs_TRDMT1_1_SG, QT00049896). The housekeeping gene *GAPDH* was amplified using the primers *GAPDH_fwd* (ATCCACCCATGGCAAATTC) and *GAPDH_rev* (TCTCGCTCTGGAAGATGGT). Using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference, relative quantification was performed using the E-method of the LightCycler 480 software (Roche). Primer specific efficiencies were calculated from serial cDNA dilutions using the second derivative maximum method.

For protein expression analysis, 50 µg of protein extracts were separated on a 10% SDS-polyacrylamide gel, followed by immunoblotting on nitrocellulose and visualization using an anti-DNMT2 antibody (Santa Cruz SC-20702, 1:1000). For RNAi experiments, knockdown of DNMT2 was performed using On-Target plus SMART pool siRNA (Dharmacon). On-Target plus siControl nontargeting siRNA (Dharmacon) was used as a control. Cells were transfected with 100 nmol/L siRNA using DharmaFect

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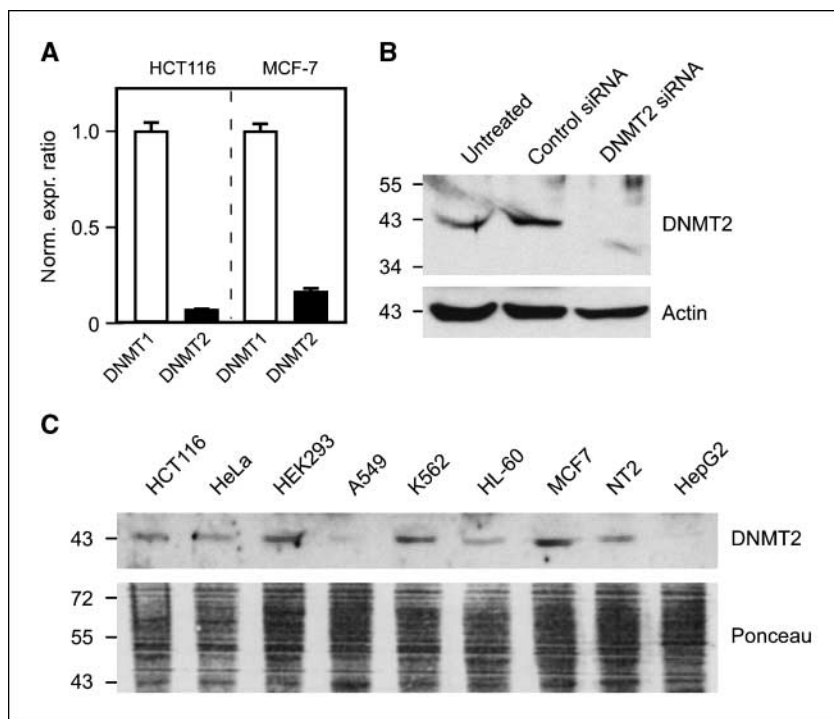


Figure 1. Expression of DNMT2 in human cancer cell lines. *A*, quantitative RT-PCR analysis of DNMT2 and DNMT1 mRNA expression in HCT116 and MCF-7 cells. CP values were <25 in all analyses. GAPDH expression was used as a reference and expression levels were normalized to DNMT1 (= 1). *Columns*, means from three experiments; *bars*, SD. *B*, DNMT2 protein is expressed in HCT116 cells. Antibody specificity was confirmed by siRNA knockdown of DNMT2. *C*, Western analysis of DNMT2 expression in protein extracts from various human cancer cell lines. The Ponceau-stained membrane was used as a loading control because individual cell lines showed different expression levels of commonly used loading control proteins.

4 (Dharmacon) and subsequently cultured for 72 h, according to the manufacturer's instructions.

tRNA bisulfite sequencing. RNA bisulfite sequencing of tRNA^{Asp} was performed as described previously (14), with the following modifications: tRNA^{Asp} was reverse transcribed using either the reverse transcription stem-loop primer 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTGGC-TCCCA-3' (tRNA sequence indicated in *italics*) or random hexamers. cDNA was PCR amplified using the bisulfite primers 5'-TAGTATAGTGGTGGTATT-3' (forward) and 5'-CGGCAATTCAGTTGAG-3' (reverse for stem loop primed) or 5'-CTCCCATCAAAAATTA-3' (reverse for hexamer primed). 28S rRNA methylation was analyzed using the reverse transcription primer 5'-ATCTAAACCAACTCACA-3' and the bisulfite primers 5'-TTTAAAGTAGGAGTGT-3' (forward) and 5'-ATCTAAACCAACTCACA-3' (reverse). For 454 sequencing, bisulfite sequence-specific PCR amplicons were gel purified and reamplified using primer pairs containing 454 linker sequences.

Metabolic activity assay. Cells were seeded into multiwell plates. Twenty-four hours after seeding, cells were treated with various concentrations of azacytidine and decitabine, respectively, for 24 h. Metabolic activity was determined by the CellTiter-Blue assay (Promega) for HCT116 cells and by the CellTiter-Glo assay (Promega) for HL-60, K562, and ML-1 cells. Assays were performed according to the manufacturer's instructions. Standard curves for dose response analysis were plotted in SigmaPlot, version 10.0 (Systat Software) using the four-parameter logistic function. Functions were plotted only when metabolic activity was reduced sufficiently to meet the "s" shape criterion for the concentration range used in this assay.

DNA methylation analysis. Genomic DNA was purified using the DNeasy Blood and Tissue kit (Qiagen). Global methylation levels were determined by capillary electrophoresis, as described previously (15).

Results

The cytidine analogue azacytidine has found increasing use for the clinical treatment of myeloid leukemias (2). Although it is generally assumed that azacytidine acts through covalent trapping of DNMT enzymes and inhibition of DNA methylation, the mode of action of this drug is not clearly understood (3). Two previous studies have suggested that azacytidine also affects tRNA methylation (16, 17), but these observations have never been followed up in detail. However,

these findings, together with the observation that DNMT2 catalyzes the methylation of tRNA^{Asp} (8), raised the possibility that DNMT2 might be inhibited by azacytidine.

The relevance of DNMT2 for tumorigenesis has not been investigated yet. DNMT2 mutant mice do not display obvious mutant phenotypes (8) and have not been tested for their tumor susceptibility. Because mouse DNMT2 has been shown to be predominantly expressed in testis (18), we needed to establish DNMT2 expression in human cancer cell lines. As an initial step, we used quantitative RT-PCR to determine DNMT2 mRNA expression in HCT116 colon carcinoma and MCF-7 breast cancer cells. The results showed that DNMT2 mRNA is expressed in both cell lines, but at lower levels than DNA methyltransferase 1 (Fig. 1A). We also analyzed DNMT2 protein levels by Western analysis of protein extracts from HCT116 cells using DNMT2-specific antibodies. This revealed a polypeptide of ~40 kDa that could be effectively depleted after transfection of the cells with siRNAs directed against DNMT2, thus confirming the specificity of the antibody (Fig. 1B). DNMT2 protein expression levels were subsequently analyzed in a panel of human cancer cell lines representing different tumor entities and different tissues of origin. The results showed variable DNMT2 protein expression in these cell lines (Fig. 1C), with comparably high levels in K562 and MCF7 cells and very low levels in A549 and HepG2 cells.

A previous study has established cytosine 38 (C38) of tRNA^{Asp} (Fig. 2A) as the primary substrate of DNMT2 in various model systems (13). To determine the tRNA^{Asp} methylation pattern in HCT116 cells, we used RNA bisulfite sequencing (14). Sequencing of 11 independent clones from control HCT116 cells showed that tRNA^{Asp} was methylated at C38, C48, and C49 (Fig. 2B). When cells were transfected with control siRNAs, this methylation pattern was not significantly affected (Fig. 2B). Sequencing of 14 independent clones from HCT116 cells that had been transfected with siRNAs directed against DNMT2 showed a complete loss of methylation at C38 (Fig. 2C), consistent with the notion that C38 is methylated by DNMT2 (13). Interestingly, the results also indicated reduced levels

of methylation at C48 (Fig. 2C), a residue that has been considered to be methylated by the TRM4 family of tRNA methyltransferases (19). This finding raises the possibility that DNMT2 can also methylate C48 in tRNA^{Asp}, or that the methylation at this position is indirectly influenced by the methylation at C38. In contrast, C49 methylation, which is catalysed by the TRM4 family of proteins (19), was not affected in cells lacking DNMT2 (Fig. 2C). Our results thus confirm a role of DNMT2 in the site-specific methylation of tRNA^{Asp} in human cancer cells.

In subsequent experiments, we used the same methodology to test for effects of azacytidine on tRNA^{Asp} methylation. When HCT116 cells were treated with 1 $\mu\text{mol/L}$ azacytidine for 1 day, methylation of C38 was considerably reduced, whereas methylation of C49 seemed unchanged (Fig. 2D). Of note, parallel treatment with 1 $\mu\text{mol/L}$ decitabine did not cause loss of methylation in tRNA^{Asp} (Fig. 2D), and the difference in C38 methylation between the azacytidine- and decitabine-treated samples was statistically significant ($P = 0.038$, Fisher's Exact test). This indicates that the ribonucleoside azacytidine is more effective in inhibiting C38 methylation of tRNA^{Asp} than the deoxyribonucleoside decitabine.

Azacytidine and decitabine are widely considered to have similar modes of action. However, differences in the cellular effects of these drugs have also been shown (20). tRNA methylation is generally assumed to regulate tRNA functionality and thereby influences the metabolic activity of cells. The selective inhibition of tRNA methylation by azacytidine thus raised the possibility that azacytidine and decitabine might also show differential effects on

the metabolic activity of human cancer cell lines. We therefore treated HCT116 cells with various concentrations of azacytidine or decitabine for 24 hours and determined the metabolic activity of the cells. The results showed that azacytidine caused a distinct drug response at low micromolar concentrations, whereas decitabine showed a similar effect only at 50-fold higher concentrations (Fig. 3). We also performed metabolic activity assays in a set of three cell lines (HL-60, K562, and ML-1) representing myeloid leukemias, the most frequent indication for the clinical use of azacytidine. All three cell lines showed a clear response to azacytidine, and a substantially weaker response to decitabine (Fig. 3). These results are consistent with the differential tRNA methylation inhibition activities of azacytidine and decitabine and suggest that inhibition of RNA methylation might be an important aspect in the cellular response to azacytidine.

Having shown that azacytidine affects the metabolic activity of myeloid leukemia cells, we also sought to confirm azacytidine-mediated demethylation of tRNA^{Asp} in these cell lines. Bisulfite sequencing of tRNA^{Asp} from control K562 cells showed a methylation pattern that was similar to control HCT116 cells, with methylation at C38, C48, and C49 (Fig. 4A). Treatment with 1 $\mu\text{mol/L}$ azacytidine for 3 days caused significant ($P < 0.001$, Fisher's Exact test) demethylation at C38 and also at C48 (Fig. 4A). To confirm this observation in an additional myeloid leukemia cell line and to analyze the specificity of azacytidine-induced demethylation in greater detail, we used 454 technology to sequence tRNA^{Asp} and 28S rRNA in HL-60 cells. This approach

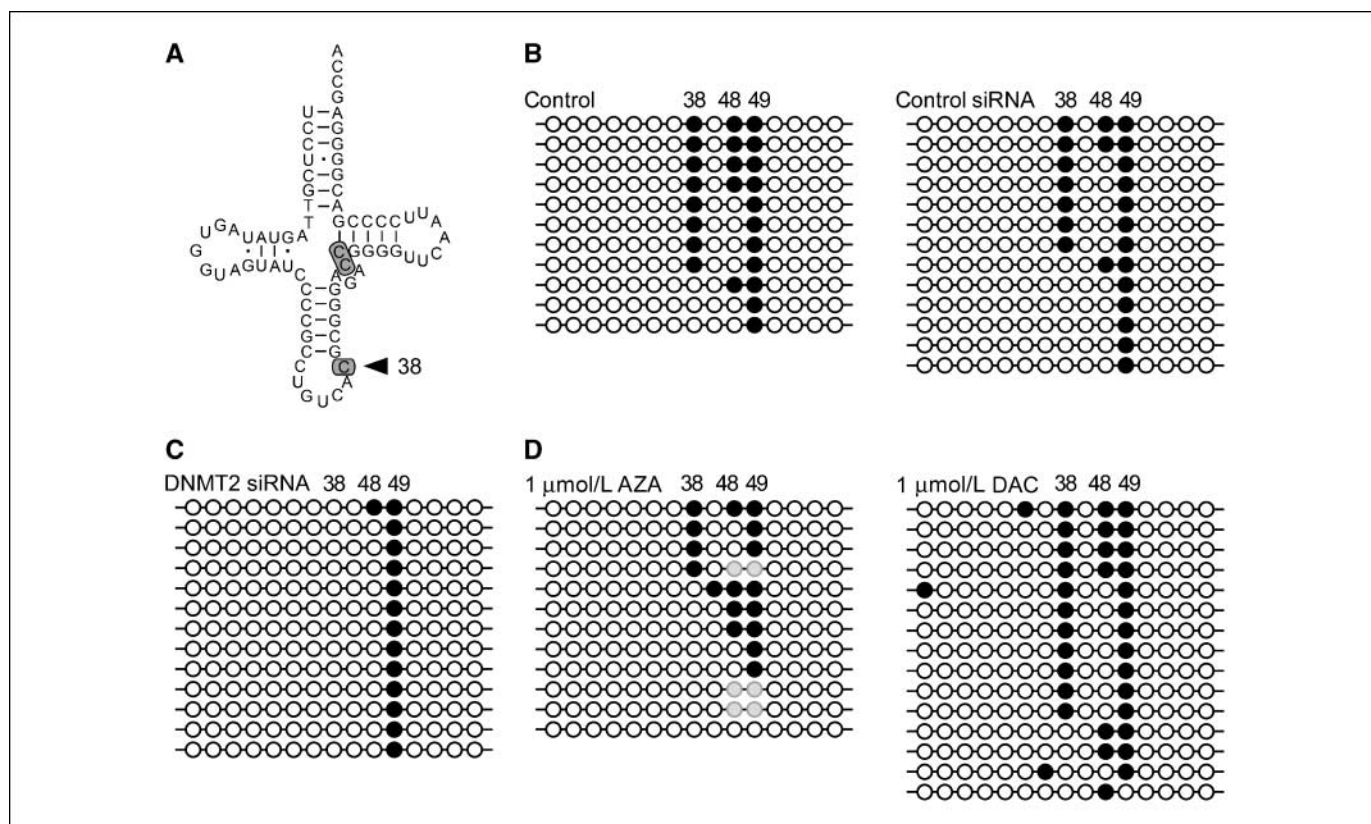


Figure 2. Azacytidine induces tRNA^{Asp} hypomethylation at DNMT2 target sites in HCT116 cells. *A*, sequence and structure of tRNA^{Asp}. *Gray shades*, methylated cytosine residues (C38, C48, C49); *arrowhead*, the known C38 target site of DNMT2. *B*, RNA bisulfite sequencing analysis of tRNA^{Asp} in HCT116 cells (*Control*) and in HCT116 cells transfected with control siRNAs. *C*, RNA bisulfite sequencing analysis of tRNA^{Asp} in HCT116 cells transfected with DNMT2 siRNAs. *D*, RNA bisulfite sequencing analysis of tRNA^{Asp} in HCT116 cells treated with 1 $\mu\text{mol/L}$ azacytidine (AZA) or decitabine (DAC), respectively, for 1 d. *Black circles*, methylated cytosine residues; *white circles*, unmethylated cytosine residues; *gray circles*, sequencing gaps.

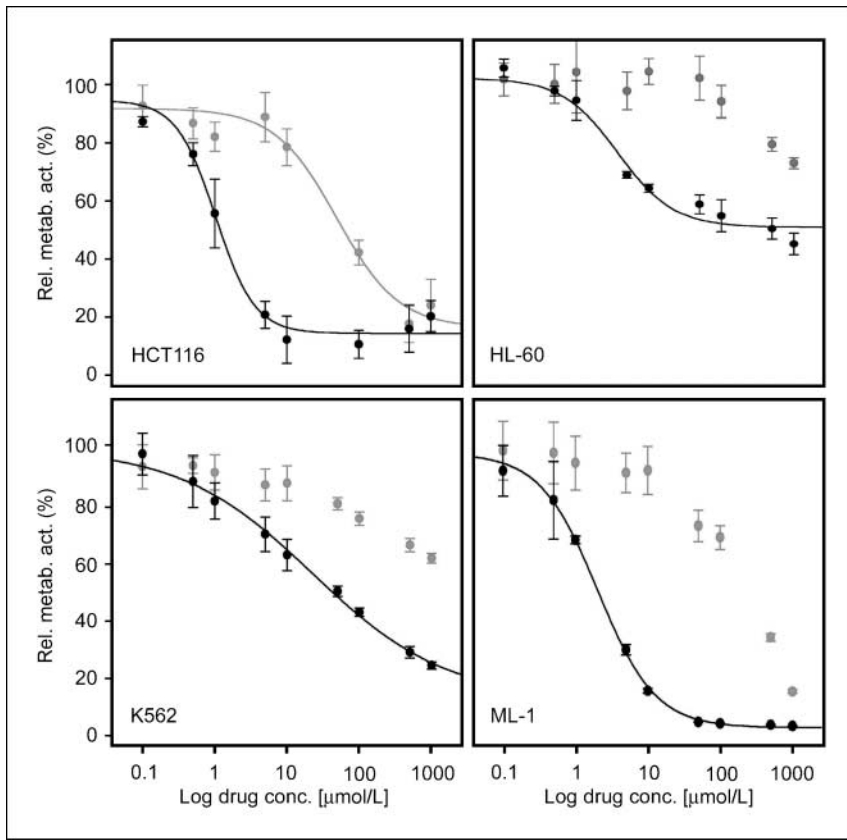


Figure 3. Azacytidine inhibits metabolic activity in human cancer cell lines. Metabolic activity was determined in various cell lines treated with azacytidine (black symbols) or decitabine (gray symbols). Dose response curves are indicated, if the data met the criteria for standard dose response curve analysis (see Materials and Methods for details). Each measurement was performed at least thrice; points, mean; bars, SD. *Rel. metab. act.*, relative metabolic activity.

allowed us to analyze four methylation sites modified by three distinct RNA methyltransferases (Fig. 4B). The evaluation of several hundred sequence reads revealed that tRNA^{Asp} from HL-60 cells was methylated at C38, C48, and C49 (Fig. 4C), in agreement with

the methylation pattern observed in other cell lines. Treatment with 500 nmol/L azacytidine for 3 days caused substantial demethylation of C38. Similar effects were also observed at C48, which is consistent with our results obtained in K562 cells and after

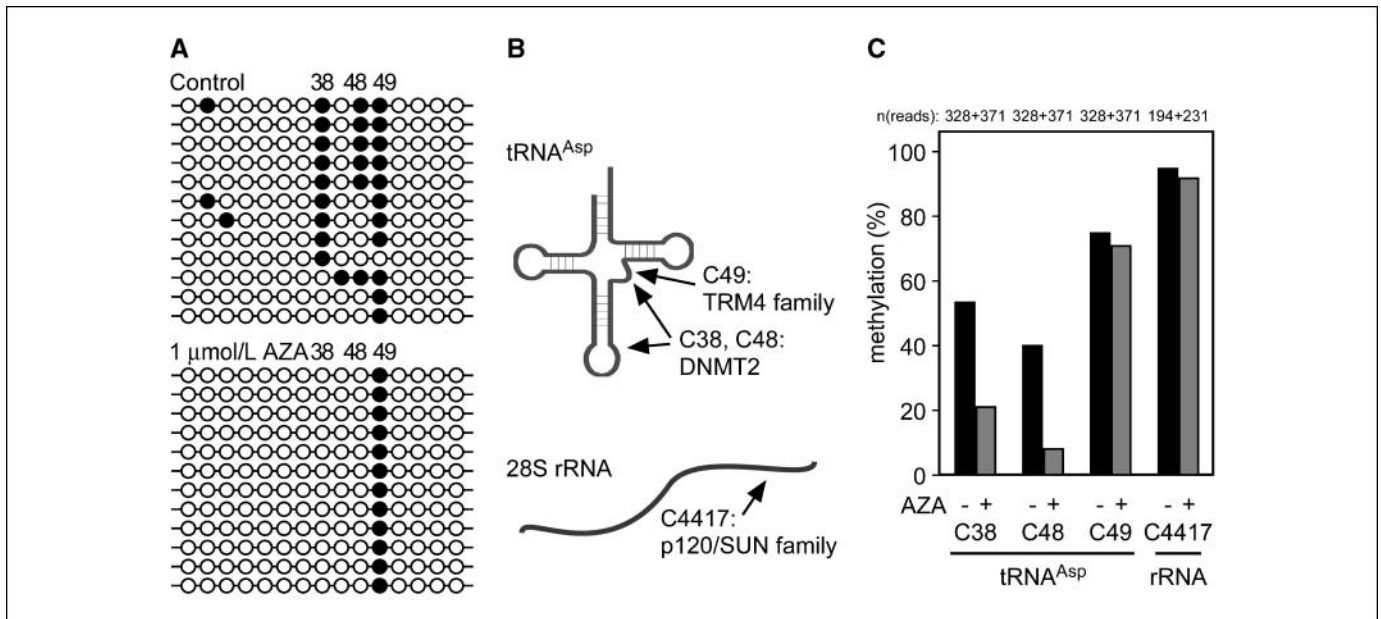


Figure 4. Azacytidine inhibits tRNA methylation at DNMT2 target sites in myeloid leukemia cells. **A**, RNA bisulfite sequencing analysis of tRNA^{Asp} in K562 cells (Control) and in K562 cells treated with 1 μmol/L azacytidine for 3 d (AZA). **B**, schematic illustration of the target sites of various tRNA and rRNA methyltransferases. **C**, 454 bisulfite sequencing analysis of tRNA^{Asp} in HL-60 cells. Columns, methylation levels of individual cytosine residues from tRNA^{Asp} and 28S rRNA, as determined by 454 bisulfite sequencing. Black columns, methylation levels from control HL-60 cells; gray columns, methylation levels from HL-60 cells treated with 500 nmol/L azacytidine for 3 d. Numbers above columns, the number of sequence reads.

siRNA-mediated knockdown of DNMT2 in HCT116 cells. In contrast, C49 methylation, which is mediated by the TRM4 family of proteins (19), was not affected by azacytidine (Fig. 4C). Similarly, C4417 of 28S rRNA, which is methylated by a member of the p120/SUN family of RNA methyltransferases (19), did not show any azacytidine-dependent methylation changes (Fig. 4C). This demethylation pattern is consistent with a selective inhibition of DNMT2 activity.

To further characterize azacytidine-mediated loss of tRNA methylation, we also compared tRNA demethylation and DNA demethylation dose responses. To this end, HL-60 cells were treated for 3 days with various concentrations of azacytidine and C38 methylation levels of tRNA^{Asp} were determined by the analysis of 100 to 600 sequence reads per data point. In parallel analyses, genomic cytosine methylation levels were determined by capillary electrophoresis. The results confirmed a concentration-dependent demethylation of C38 from 54% in untreated control cells to 21% in cells treated with 500 nmol/L azacytidine (Fig. 5A). At higher drug concentrations, C38 methylation levels were similar to control levels (Fig. 5A). It is possible that higher drug concentrations cause a general inhibition of the RNA metabolism and a reduction in the synthesis of new and unmodified tRNA molecules. In contrast to the clearly defined concentration optimum for tRNA^{Asp} demethylation at 500 nmol/L, significant DNA demethylation could only be observed at the highest test concentration (1 μ mol/L; Fig. 5A), thus

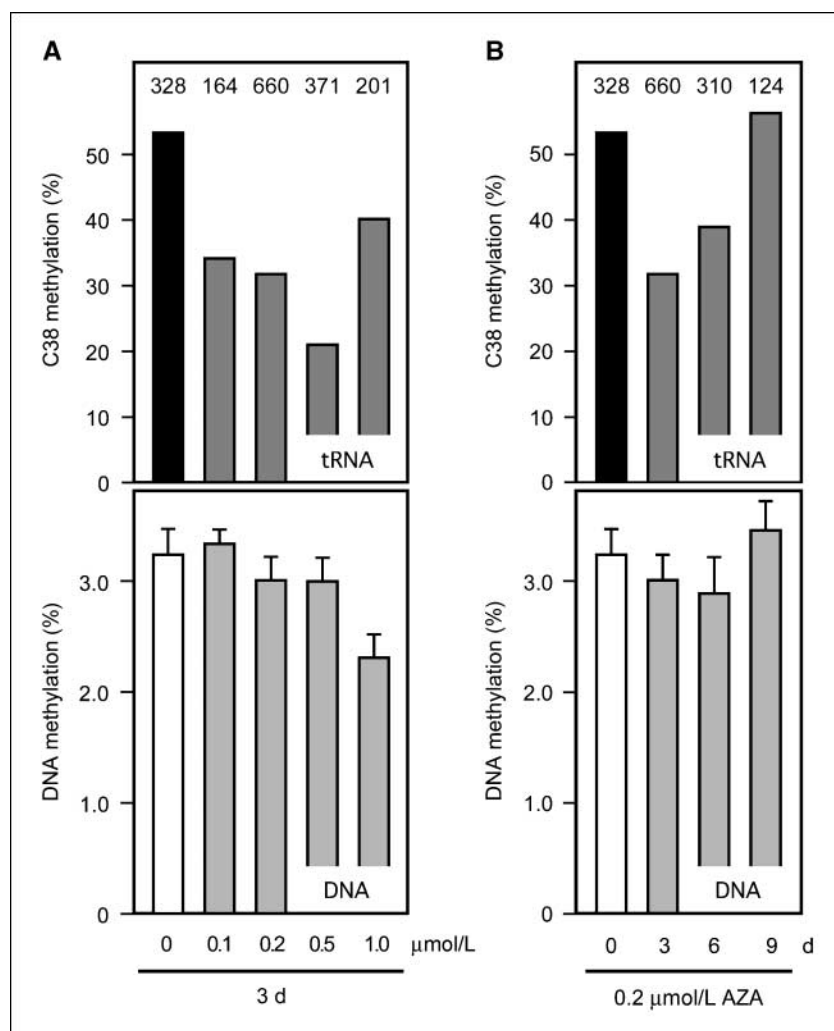
indicating that tRNA^{Asp} is more sensitive to azacytidine-induced demethylation than DNA.

We also analyzed how repeated 3-day treatment cycles with low concentrations (200 nmol/L) of azacytidine affect tRNA^{Asp} methylation. C38 demethylation was readily detectable after the first treatment cycle (Fig. 5B). This effect was lost after the third treatment cycle, when methylation levels were similar to control levels (Fig. 5B). When we used capillary electrophoresis to determine genomic DNA methylation levels under the same conditions, we did not observe any significant DNA demethylation, even after three treatment cycles (Fig. 5B). These findings further illustrate the sensitivity of tRNA^{Asp} methylation to azacytidine and establish (de)methylation of tRNA^{Asp} as a novel molecular biomarker for azacytidine responses in human cancer cell lines. The reasons for the inability of azacytidine to demethylate tRNA^{Asp} after repeated drug incubation are presently unclear and will have to be investigated in future studies. It is possible that repeated doses of low azacytidine concentrations cause a general inhibition of the RNA metabolism, comparable with the effects observed after a single dose with high drug concentrations.

Discussion

The role of the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B in tumorigenesis has been extensively analyzed (21).

Figure 5. Comparison of azacytidine-dependent tRNA^{Asp} demethylation and DNA demethylation in HL-60 myeloid leukemia cells. *A*, cells were treated with various concentrations of azacytidine as a single dose for 3 d. 454 bisulfite sequencing was used to determine the methylation level of C38 in tRNA^{Asp}, or capillary electrophoresis was used to determine global DNA methylation levels. *B*, for repeated treatment, cells were splitted every 3 d in medium containing a low concentration (200 nmol/L) of azacytidine. Demethylation of tRNA^{Asp} was detectable only after the first two treatment cycles. No significant DNA demethylation could be observed under these conditions. *Numbers above columns*, numbers of sequence reads. Capillary electrophoresis measurements were repeated at least thrice; *columns*, mean; *bars*, SD.



In contrast, there are no published studies on the role of the fourth member of this family, DNMT2, in human cancers. DNMT2 has been shown to have a prominent tRNA methyltransferase activity in various model systems (13), which separates this enzyme from other DNMT proteins. In addition, this finding also raises the possibility that altered DNMT2 activity might contribute to tumorigenesis through novel pathways that are related to RNA methylation. Although the precise function of DNMT2-mediated RNA methylation remains to be established, tRNA methylation has been implied in the regulation of tRNA folding and stability (22, 23). It is conceivable that misfolded and/or less stable tRNAs might impact the rate of protein synthesis in cancer cells. Our recent analysis of tRNA methylation patterns in DNMT2 mutant *Drosophila melanogaster* has uncovered additional enzyme substrates,¹ which suggests that the translation of several codons might be affected by the inhibition of DNMT2-mediated methylation.

Azacytidine has found widespread laboratory use as an inhibitor of DNA methylation and our results show that the drug can also inhibit cytosine methylation in tRNA^{Asp} with considerable specificity. Our bisulfite sequencing results revealed efficient demethylation of tRNA^{Asp} at C38 and at C48, thus establishing two sites as biomarkers for monitoring DNMT2 function during or after azacytidine treatment. Other methylated cytosines, such as C49 of tRNA^{Asp}, and C4417 of 28S rRNA, which are methylated by other RNA methyltransferases, were only weakly affected by azacytidine. The use of a DNA methyltransferase-like mechanism for the methylation of tRNA substrates (24) might provide an explanation for the particular sensitivity of DNMT2 to azacytidine. Because our study only analyzed a limited number of RNA methylation sites, we cannot exclude the possibility that other RNA methyltransferases are also inhibited by azacytidine. The detection

of such effects might also have been obscured by general drug effects on RNA metabolism, thus resulting in a reduced representation of newly synthesized RNA molecules. In this scenario, the bisulfite sequencing approach would only allow us to recover RNA molecules that had been synthesized and methylated before drug treatment.

Regardless of the precise mechanism of tRNA methylation inhibition, our results may prove to be useful for the clinical development of azacytidine. Over the past few years, azacytidine, and its deoxynucleoside variant, decitabine, have been developed as archetypal drugs for epigenetic cancer therapy (25). Both drugs have shown to be similarly effective in inducing DNA demethylation in cancer cell lines and in patients (6–9, 20), which has fostered the notion that the mode(s) of action of both drugs are highly similar. Our results indicate that azacytidine is more effective than decitabine in inhibiting tRNA methylation, and thus provide an important example for a cellular pathway that is selectively affected by one of the drugs. In addition, our results also suggest that azacytidine-induced tRNA demethylation can be detected with higher sensitivity than azacytidine-induced DNA demethylation. It will be important to characterize the effects of azacytidine on RNA methylation patterns in patients undergoing azacytidine therapy and to evaluate RNA methylation markers for their potential as molecular markers predicting clinical responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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