Published in final edited form as:

Biochemistry. 2017 April 25; 56(16): 2166–2169. doi:10.1021/acs.biochem.7b00208.

DNA Methyltransferases Demonstrate Reduced Activity Against Arabinosylcytosine: Implications for Epigenetic Instability in AML

Christopher S. Nabel^{†,§}, Jamie E. DeNizio^{†,§}, Martin Carroll[†], and Rahul M. Kohli^{†,§,*}

[†]Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

[§]Department of Biochemistry & Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

Abstract

Arabinosylcytosine (araC) is a mainstay in the initial treatment of acute myeloid leukemia (AML), although relapses are common. Given recent recognition of altered DNA methylation patterns in relapsed AML, we considered whether araC, which acts by incorporation into DNA, could itself perturb methylation dynamics. To explore this possibility, we examined several DNA methyltransferases and find that araC embedded in DNA is consistently methylated at diminished efficiency relative to deoxycytidine (dC). Importantly, with the human maintenance methyltransferase DNMT1, araC methylation is reduced by more than ~200-fold. These observations support a model whereby araC treatment may itself contribute to locus-specific, passive DNA demethylation in relapsed AML.

TOC image

*Corresponding Author: Rahul M. Kohli, Department of Medicine, Department of Biochemistry & Biophysics, Perelman School of Medicine; University of Pennsylvania, 502B Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104, Tel.: (215) 573-7523; b.kohli@upenn.edu.

Supporting Information

The supporting information includes Experimental Procedures, as well as Table S1 and Figure S2.

Author Contributions

CSN conceived of the study, performed experiments in Figures 1 and 2, analyzed results, and wrote the manuscript. JED performed experiments in Figure 3, analyzed results, and contributed to the writing of the manuscript. MC and RMK conceived of the study, and both helped to analyze results and to write the manuscript. All authors approved the final version of the manuscript.

The authors declare no competing financial interests.

Keywords

DNA Methyltransferase; epigenetics; leukemia; 5-methylcytosine; DNA demethylation; cytarabine; epigenetic instability; chemoresistance

Emerging cancer treatments are deeply rooted in mechanism, aiming to achieve targeted and rational inhibition of pathways driving tumorigenesis. This paradigm deviates from early strategies in cancer treatment, where anti-metabolites were identified and found to have clinical efficacy without the prerequisite for insight into their mechanisms of action. Arabinosylcytosine (araC), a present-day mainstay of therapy for acute myeloid leukemia (AML), emerged from this earlier paradigm. First synthesized in the late 1950s, araC was initially identified as an anti-metabolite with potent *in vitro* activity against viruses^{1,2} and cancer cell lines.^{3–5} Shortly thereafter, araC received approval for treatment in AML despite an incomplete understanding of how leukemic cell killing is achieved. Today, the agent remains incorporated in virtually all regimens for induction chemotherapy.^{6,7}

AraC differs from deoxycytosine (dC) by the addition of a single 2'-hydroxyl group in the sugar, in an epimeric configuration opposite that of ribose. In the years following the discovery of its clinical efficacy, much of araC's mechanism of action has been elucidated. After being taken up by cells, araC is processed to the nucleotide triphosphate (araCTP).^{8,9} AraCTP, then, can act by two mechanisms to alter the activity of DNA polymerases. First, araCTP can prevent incorporation of natural nucleotide triphosphates into DNA by acting as a competitive inhibitor.^{10,11} Second, araCTP can itself function as a substrate for these DNA polymerases, resulting in the incorporation of araC lesions within the genome. While some early biochemical studies suggested that araC incorporation results in chain termination,^{10,12} later studies have convincingly demonstrated that araC does not completely block elongation and remains embedded in DNA.^{13,14} Further, in some repair processes, the DNA polymerase employed, such as DNA polymerase β , have a particular propensity to incorporate and extend past araC in genomic DNA.^{15–17}

AraC's incorporation into genomic DNA is particularly relevant in light of recent findings regarding the importance of epigenetics in AML. In one study on a cohort of 140 AML patients, upon relapse after araC treatment, only minimal new genetic changes were identified; however, variability in DNA methylation at clusters of cytosine-guanine dinucleotides (CpGs), the predominant site of methylation, increased significantly after araC treatment.¹⁸ The finding that epigenetic, rather than genetic, changes predominate in relapsed AML raises the question of how araC, as an analog of dC in DNA, might impact DNA methylation machinery. This machinery includes both the DNMT3 methyltransferases that initiate methylation *de novo*, as well as the maintenance methyltransferase DNMT1, responsible for upkeep of methylation patterns during cellular division and DNA repair.¹⁹ To evaluate whether araC could itself contribute to changing epigenetic patterns in AML, we examined reactivity of DNA methyltransferases (MTases) against araC incorporated into synthetic DNA substrates, a question that to our knowledge has not been previously explored.

Accordingly, we first developed an assay to screen for the generation of arabinosyl-5-methylcytidine (ara-5mC) within DNA. To this end, we sought to use methylation-sensitive restriction enzymes (MSREs), which have long been used to differentiate methylated from unmethylated DNA. MSREs exhibit strong cleavage activity against dC, but are blocked by deoxy-5-methylcytidine (d-5mC), permitting separation of methylated and unmethylated DNA by gel electrophoresis. We have previously shown that certain restriction endonucleases are capable of cleaving synthetic substrates containing a single chimeric araC nucleotide within an otherwise deoxy-containing DNA backbone.²⁰ To test whether some MSREs might also possess activity against chimeric DNA substrates containing araC and retain their ability to discriminate between methylation states (araC vs ara-5mC), we designed a series of substrates. The experimental assay strand (AS1a) contains araC embedded within the CpG dinucleotide recognition site for human MTases; the CpG exists within the larger recognition sequence of various possible restriction endonucleases (Fig. 1A). The corresponding control assay strands contain dC (AS1c) or its associated product control d-5mC (AS1m). For MTase reactions, the assay strand was annealed to a complementary strand (AC1) containing a corresponding d-5mC within the palindromic recognition sequence. By using a methylated complement strand, we aimed to ensure that MTase activity was confined to the assay strand of the duplex. After MTase treatment, the AS/AC duplexes were melted and re-annealed with excess of a complementary strand containing unmethylated dC, referred to as the digestion complement (DC1) (See Supporting Information for further details). After screening numerous potential MRSEs (Table S1), we found that BsrFI exhibited the desired discriminatory activities. As part of its canonical activity, BsrFI cleaves DNA containing dC, but not d-5mC (Fig. 1B, C). When araC replaces dC in the target sequence, BsrFI can still cleave the DNA, making it feasible that it might discriminate against ara-5mC.

We hypothesized that under favorable conditions, a proficient DNA MTase could methylate substrates containing araC, and that these products would be resistant to BsrFI cleavage. To examine this possibility, we initially focused on the highly active bacterial MTase HpaII (M.HpaII). We treated our araC-containing substrate with M.HpaII under excess conditions that resulted in complete methylation of dC within its canonical CCGG recognition site (Fig. 1C). Under these conditions, the araC-containing DNA was also now protected from cleavage by BsrFI, suggesting enzymatic methylation of araC (Fig. 1C). To confirm that protection from BsrFI cleavage truly represents enzymatic methylation of araC, the masses of these oligonucleotides were analyzed by electrospray ionization mass spectrometry before and after reaction with M.HpaII (Fig. 1D). M.HpaII treatment resulted in the loss of the peak corresponding to the unmodified araC-containing substrate and the appearance of a new peak with a mass increase consistent with methylation. Thus, M.HpaII is capable of methylating araC in DNA, and the methylation-sensitive restriction endonuclease BsrFI can discriminate between ara-5mC and araC.

Having observed activity that araC can be methylated *in vitro*, we wanted to examine whether this assay could be used to examine the relative efficiency of activity on araC compared to dC. We evaluated the reactivity of M.HpaII with substrates containing either dC or araC by titrating enzyme concentration over a range of activity. Incubation with equivalent amounts of M.HpaII protected the dC-containing substrate from BsrFI cleavage

to a greater extent than the araC-containing substrate, indicating that dC is a better substrate for methylation (Fig. 2A).

Having established a reliable assay for araC methylation and detection, we next focused on the clinically relevant question of human MTase reactivity, given the potential implications for epigenetic instability in AML relapse after treatment. We incubated either the purified *de novo* MTase DNMT3A/3L or maintenance MTase DNMT1 with cytosine-containing substrates. We again titrated enzyme concentration over a range of catalytic activity and analyzed relative methylation efficiency by measuring protection of the DNA from BsrFI cleavage. As with M.HpaII, both DNMT3A/3L and DNMT1 exhibit diminished methylation activity against araC-containing substrates, though to differing extents (Figs. 2B, 2C). DNMT3A/3L shows detectable activity on araC, although reduced relative to dC (Fig. 2B). Discrimination was even more apparent with DNMT1, where no significant protection from digestion was observed at even the highest enzyme concentrations tested (Fig. 2C). These results show a consistent preference for dC over araC and demonstrate that the extent of this preference can vary considerably, with a greater discrimination seen by maintenance machinery (DNMT1) than initiation machinery (DNMT3A/3L) among human enzymes.

To extend beyond qualitative observations offered by our MRSE-based assay, we aimed to quantify the differential preferences of DNMT1 and DNMT3A/3L for methylating araC. For quantitative measurement and sensitive detection of methylation, we utilized a radiolabel-based assay (Fig. 3A). Assay strands containing a single CpG as either araC (AS2a) or the corresponding control with dC (AS2c) were generated and duplexed to a biotinylated complementary strand containing d-5mC in the complementary CpG (AC2). The biotinylated duplexes were incubated with MTases in the presence of ^3H -S-adenosylmethionine, the reactions were quenched at time points up to 30 min, and the DNA was purified using streptavidin-coated magnetic beads. The product DNA was then analyzed for ^3H -content by liquid scintillation counting (See Supporting Information for further details).

Under these conditions, DNMT3A/3L incubation revealed linear, time-dependent incorporation of ^3H into dC-containing DNA (Fig. 3B). The activity on araC-containing DNA was also linear and time-dependent, with a 3.7-fold reduction relative to dC. As with DNMT3A/3L, DNMT1 also showed time-dependent activity against dC-containing DNA (Fig. 3C). Consistent with our qualitative result with DNMT1, we observed no detectable incorporation of ^3H into the araC-containing DNA at early time points, with levels only detectable above background at 30 min. Given the limit of detection of our assay, our results indicate that DNMT1 is more than 200-fold more active on dC than araC, suggesting that araC incorporated into DNA could prevent routine maintenance of DNA methylation.

While araC is a mainstay of induction chemotherapy for AML, resistance often arises and can be associated with relapse.^{18,21} The observation of altered DNA methylation at relapse, independent of the somatic mutational burden, prompts the unanswered question of how this phenomenon may arise.

To our knowledge, this report is the first characterization of DNA MTase reactivity on araC-containing DNA. Although our data not address the molecular mechanisms involved, structural modeling and comparison to other enzymes that act on DNA can offer some insights into the basis for discrimination against araC (discussed in Figure S1).^{20,22–25} These data linking araC to perturbed DNMT activity suggests several novel and now plausible hypotheses for how araC treatment can contribute to the altered DNA methylation in AML relapse. As noted earlier, araC is known to be stably integrated into the genome of leukemic cells.^{10,12,13} For cells that survive therapy, our data raise the possibility that these rare araCpG sites could be resistant to maintenance methylation by DNMT1, resulting in functional passive demethylation. When site-specific methylation changes occur at regions controlling expression of transcription factors, chromatin modifiers or tumor suppressors, demethylation could confer a selective advantage to growing cells, further promoting relapse and specifically facilitating acquired resistance to araC. This postulated mechanism would not result in global demethylation, but focal, site-specific methylation changes. The precedent of impaired methylation systems resulting in focal but not global methylation changes has been previously reported in DNMT3A-mutated AML, where dominant-negative R882 mutations result in hypomethylation of specific loci, including p15/CDKN2B.²⁶

A second possible source of araC-induced epigenetic instability relates to the spontaneous deamination of 5-methylcytosine (d-5mC) sites in the genome, which generates T:G mismatches.^{27,28} When unrepaired, these lesions can result in somatic transition mutations which are highly enriched in many cancers.²⁹ However, for those lesions that are repaired, as noted earlier, the polymerases that mediate the repair process have a propensity to incorporate araC.^{15–17} As these sites require DNMT1 to restore methylation after repair, locus-specific impairment in DNA methylation could therefore be introduced if araC is incorporated, rather than unmodified C.

Our biochemical approach therefore provides several novel putative mechanisms by which araC could impact DNA methylation dynamics. The hypothesis, however, is not meant to provide a full explanation for the epigenetic changes that occur in AML. Indeed, the epiallele diversity reported in relapsed AML encompasses both increases and decreases in methylation, while our mechanism only addresses the loss of methylation at some loci. Changes to other aspects of the methylation machinery and stochastic variability are also likely important in the epigenetic dynamics in AML.

Given the growing recognition of altered DNA methylation in AML and the use of therapies targeting epigenetic mechanisms, our study newly highlights the importance of more broadly considering the interplay of nucleoside anti-metabolites with DNA-modifying enzymes. Based on the precedent that our study establishes, it is feasible that the decreased activity of DNMT3A/3L on araC could alter *de novo* methylation patterns or that araC or ara-5mC could be associated with altered demethylation dynamics by perturbing the activity of TET family enzymes that normally act upon d-5mC.³⁰ Additional studies will be required to determine whether the altered reactivity of araC with DNA MTases indeed has pathological consequences in AML and whether alternative approaches could help to minimize the impact of epigenetic instability on treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jessica Schneck from GlaxoSmithKline for guidance and the laboratory of Dr. James Hoxie for equipment use.

Funding Sources

This work was funded by the Rita Allen Foundation (to RMK), the NIH (R01-GM118501 and DP2-GM105444), and training support to JED from the NIH (T32-GM07229) and the National Science Foundation.

ABBREVIATIONS

araC	arabinosylcytosine
AML	acute myeloid leukemia
dC	deoxycytidine
araCTP	arabinosylcytosine triphosphate
MTases	methyltransferases
SAM	S-adenosylmethionine
MSRE	methylation-sensitive restriction endonuclease
d-5mC	5-methyldeoxycytidine
ara-5mC	5-methylarabinosylcytosine

References

1. Rapp F. J Immunol. 1964; 93:643–648. [PubMed: 14219080]
2. Renis HE. Cancer Res. 1970; 30:189–194. [PubMed: 5466741]
3. Evans JS, Musser EA, Mengel GD, Forsblad KR, Hunter JH. Proc Soc Exp Biol Med. 1961; 106:350–353. [PubMed: 13697589]
4. Kessel D, Hall TC, Wodinsky I. Science. 1967; 156:1240–1241. [PubMed: 6025544]
5. Graham FL, Whitmore GF. Cancer Res. 1970; 30:2627–2635. [PubMed: 5530557]
6. Kremer WB. Ann Intern Med. 1975; 82:684–688. [PubMed: 1056159]
7. Lynch RC, Medeiros BC. Expert Opin Pharmacother. 2015; 16:2149–2162. [PubMed: 26364895]
8. Cohen SS. Cancer. 1977; 40:509–518. [PubMed: 328134]
9. Kufe D, Spriggs D, Egan EM, Munroe D. Blood. 1984; 64:54–58. [PubMed: 6587917]
10. Graham FL, Whitmore GF. Cancer Res. 1970; 30:2636–2644. [PubMed: 5530558]
11. Furth JJ, Cohen SS. Cancer Res. 1968; 28:2061–2067. [PubMed: 5754840]
12. Wist E, Krokan H, Prydz H. Biochemistry. 1976; 15:3647–3652. [PubMed: 952882]
13. Major PP, Egan EM, Beardsley GP, Minden MD, Kufe DW. Proc Natl Acad Sci U S A. 1981; 78:3235–3239. [PubMed: 6942429]
14. Major PP, Egan EM, Herrick D, Kufe DW. Biochem Pharmacol. 1982; 31:861–866. [PubMed: 7200770]
15. Miller MR, Chinault DN. J Biol Chem. 1982; 257:10204–10209. [PubMed: 6179938]

16. Chen YW, Cleaver JE, Hanaoka F, Chang CF, Chou KM. *Mol Cancer Res.* 2006; 4:257–265. [PubMed: 16603639]
17. Prakasha Gowda AS, Polizzi JM, Eckert KA, Spratt TE. *Biochemistry.* 2010; 49:4833–4840. [PubMed: 20459144]
18. Li S, Garrett-Bakelman FE, Chung SS, Sanders MA, Hricik T, Rapaport F, Patel J, Dillon R, Vijay P, Brown AL, Perl AE, Cannon J, Bullinger L, Luger S, Becker M, Lewis ID, To LB, Delwel R, Lowenberg B, Dohner H, Dohner K, Guzman ML, Hassane DC, Roboz GJ, Grimwade D, Valk PJ, D'Andrea RJ, Carroll M, Park CY, Neuberg D, Levine R, Melnick AM, Mason CE. *Nat Med.* 2016
19. Klose RJ, Bird AP. *Trends Biochem Sci.* 2006; 31:89–97. [PubMed: 16403636]
20. Nabel CS, Lee JW, Wang LC, Kohli RM. *Proc Natl Acad Sci U S A.* 2013; 110:14225–14230. [PubMed: 23942124]
21. Parkin B, Ouillette P, Li Y, Keller J, Lam C, Roulston D, Li C, Shedden K, Malek SN. *Blood.* 2013; 121:369–377. [PubMed: 23175688]
22. Brown JA, Suo Z. *Biochemistry.* 2011; 50:1135–1142. [PubMed: 21226515]
23. Pearl LH. *Mutat Res.* 2000; 460:165–181. [PubMed: 10946227]
24. Nandakumar J, Shuman S, Lima CD. *Cell.* 2006; 127:71–84. [PubMed: 17018278]
25. Song J, Teplova M, Ishibe-Murakami S, Patel DJ. *Science.* 2012; 335:709–712. [PubMed: 22323818]
26. Russler-Germain DA, Spencer DH, Young MA, Lamprecht TL, Miller CA, Fulton R, Meyer MR, Erdmann-Gilmore P, Townsend RR, Wilson RK, Ley TJ. *Cancer Cell.* 2014; 25:442–454. [PubMed: 24656771]
27. Lindahl T. *Nature.* 1993; 362:709–715. [PubMed: 8469282]
28. Shen JC, Rideout WM 3rd, Jones PA. *Cell.* 1992; 71:1073–1080. [PubMed: 1473145]
29. Weisenberger DJ. *J Clin Invest.* 2014; 124:17–23. [PubMed: 24382385]
30. Kohli RM, Zhang Y. *Nature.* 2013; 502:472–479. [PubMed: 24153300]

FIGURE 1.

AraC in DNA can be methylated *in vitro*. (A) Substrates used in assay. The CpG of interest is in bold and the MSRE BsrFI restriction site is underlined. (B) Restriction endonuclease-based assay for methylation detection. (C) Duplex DNA substrates (40 nM) containing dC [C], araC [A], and d-5mC [M] were incubated in the presence or absence of 0.4 U/μL of M.HpaII at 37 °C for 12 hrs and processed using the BsrFI-based assay. Additional experimental details are provided in the Supporting Information. (D) Electrospray ionization mass spectrometry of araC-containing substrates in the presence and absence of M.HpaII incubation.

FIGURE 2. DNA Methyltransferases differentially discriminate against araC. Serial dilutions of M.HpaII (A), DNMT3A/3L (B) or DNMT1 (C) were incubated at 37 °C for 30 min with substrates (40 nM) containing dC [C] or araC [A] and assayed with BsrFI.

FIGURE 3.

Mammalian DNA methyltransferases differentially discriminate against araC. (A) Substrate design and schematic of radiolabeling assay. (B, C) Time-dependent methylation using dC- and araC-containing substrates (400 nM) incubated at 37 °C for the indicated time with either DNMT3A/3L (174 nM) (B) or DNMT1 (22.7 nM) (C). Rates of product formation (nM product * nM enzyme⁻¹ * min⁻¹) were determined from triplicate measurements at each time point. Standard deviation of each value is shown and standard deviation of the associated slope is reported. The zero time points represent no enzyme addition.