Published in final edited form as:

Chem Res Toxicol. 2011 November 21; 24(11): 2040–2043. doi:10.1021/tx2003849.

Cyclophosphamide Perturbs Cytosine Methylation in Jurkat-T Cells through LSD1-mediated Stabilization of DNMT1 Protein

Jing Zhang^{a,b}, Bifeng Yuan^a, Fan Zhang^a, Lei Xiong^a, Jiang Wu^a, Sriharsa Pradhan^c, and Yinsheng Wang^{a,*}

^aSchool of Chemistry and Materials Science, Shaanxi Normal University, Xi'an 710062, P. R. China.

^bDepartment of Chemistry, University of California, Riverside, CA 92521-0403.

^cNew England Biolabs Incorporated, 240 County Road, Ipswich, MA 01938.

Abstract

Aberrant cytosine methylation is known to be associated with cancer development. Here we assessed how common cancer chemotherapeutic agents perturb cytosine methylation in Jurkat-T acute lymphoblastic leukemia cells. We tested six anti-tumor agents and found that cyclophosphamide induced the most pronounced increase in global DNA cytosine methylation after a 24-hr treatment. Long-term treatment with cyclophosphamide led to a time-dependent increase in cytosine methylation level with up to 4 days of treatment, and the extent of cytosine methylation returned to normal level after 8 days. The trend of change in DNA methylation level paralleled that of the expression level of DNMT1 protein, whereas no significant increase in DNMT1 mRNA level was observed. Previous studies showed that the stability of endogenous DNMT1 protein is regulated by lysine methylation through histone lysine methyltransferase Set7 and lysine-specific demethylase 1 (LSD1), with the methylated DNMT1 being the target for proteasomal degradation. We observed that the elevated expression of DNMT1 protein at 4 days of treatment was correlated with the increased expression of LSD1 protein and with the decreased frequency of K142 methylation in DNMT1. Taken together, our results showed that cyclophosphamide perturbed temporarily global cytosine methylation in Jurkat-T cells via regulating the lysine methylation level in DNMT1.

Introduction

In mammals, DNA cytosine methylation pattern is established and maintained by DNA (cytosine-5)-methyltransferases (DNMTs) encompassing DNMT1, DNMT3a and DNMT3b (1–3). This covalent modification of DNA, together with post-translational modifications of histone proteins and microRNA expression (4), functions as an important mediator of gene regulation and constitutes the cornerstone for the vibrant field of epigenetics. Mounting evidence indicates that the alteration in DNA methylation may be an early event during tumor development (5). Along this line, aberrant promoter cytosine methylation was found in DNA from secretions and body fluids of individuals many years prior to the clinical diagnosis of cancer (6, 7).

*To whom correspondence should be addressed: yinsheng.wang@ucr.edu; Tel.: (951)827-2700; Fax: (951)827-4713.

Supporting Information Available: Quantitative real-time RT-PCR and Western blot results. This material is available free of charge via the Internet at <http://pubs.acs.org>

The pharmacological modulation of aberrant DNA methylation patterns using hypomethylating agents such as azacytidine and decitabine was recently found to be effective in treating hematological malignancies. These drugs have demonstrated significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), a preleukemic bone marrow disorder (8, 9). Our recent study also demonstrated that treatment with 6-thioguanine, an agent used in anti-leukemia therapy, could lead to global cytosine demethylation and reactivation of epigenetically silenced genes in acute lymphoblastic leukemia cells (10). Moreover, an earlier study showed that several commonly used cancer chemotherapeutic agents could perturb global DNA cytosine methylation at toxic concentrations (11). However, it has not been investigated how cytosine methylation pattern in human tumor cells is perturbed upon treatment with therapeutically relevant concentrations of these drugs. In the present study, we treated Jurkat-T cells with six commonly used cancer chemotherapeutic agents, including 1- β -D-arabinofuranosylcytosine (arabinose C), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), cisplatin, cyclophosphamide, doxorubicin and etoposide (structures depicted in Figure S1), at therapeutically relevant concentrations and assessed global cytosine methylation levels in these cells before and after the drug treatment. Our results revealed that cyclophosphamide could lead to a transient increase in DNA methylation level through augmenting the expression level of DNMT1 protein, which is in turn regulated by the elevated expression of lysine-specific demethylase 1 (LSD1).

Experimental Procedures

Chemicals and Enzymes

All chemicals and enzymes, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO). Jurkat-T (clone E6-1) acute lymphoblastic leukemia cells, penicillin, streptomycin, and RPMI-1640 media were purchased from ATCC (Manassas, VA). *Cell Culture, Drug Treatment and DNA Isolation.* Jurkat-T cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and penicillin (100 IU/mL). Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C, with medium renewal at every 2–3 days when cells reached 60% confluence. Cells were treated with 0, 3 or 10 μ M drug for 24 hr in fresh media. For long-term cyclophosphamide treatment, the media were changed and freshly prepared drug was added daily, and cells were harvested at 1, 2, 4, 5, 8 and 10 days for DNA extraction.

DNA Isolation

The untreated and treated cells ($\sim 2 \times 10^7$ cells) were harvested by centrifugation (500 g). The cell pellets were washed with phosphate-buffered saline (PBS) and resuspended in a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, and 0.5% SDS. The cell lysates were then treated with 20 μ g/mL RNase A at 37°C for 1 hr and subsequently with 100 μ g/mL proteinase K at 50°C for 3 hr. Genomic DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and desalted by ethanol precipitation. The DNA pellet was redissolved in water, and its concentration was determined by UV absorption measurement.

DNA Digestion and HPLC Quantification of Global Cytosine Methylation

The isolated cellular DNA (50 μ g) was denatured by heating to 95°C and chilled immediately on ice. The denatured DNA was digested with 2 units of nuclease P1 in a buffer containing 30 mM sodium acetate (pH 5.5) and 1 mM zinc acetate at 37°C for 4 hr. To the digestion mixture was added 12.5 units of alkaline phosphatase in a 50 mM Tris-HCl buffer (pH 8.6). The digestion was continued at 37°C for 3 hr, and the enzymes were removed by chloroform extraction. The aqueous DNA layer was lyophilized by Speed-Vac, and the dried DNA was redissolved in water. The digested nucleoside mixture was quantified by UV

absorbance measurements, and subjected to HPLC separation on an Agilent 1100 capillary pump (Agilent Technologies, Palo Alto, CA) with a UV detector monitoring at 260 nm and a Peak Simple chromatography data system (SRI Instruments Inc., Las Vegas, NV). A 4.6×250 mm Polaris C18 column (5 μ m in particle size; Varian Inc., Palo Alto, CA) was used for the separation of the enzymatic digestion mixture. A solution of 10 mM ammonium formate (pH adjusted to 4.0 with the addition of HCl, solution A) and a mixture of 10 mM ammonium formate and acetonitrile (70:30, v/v, solution B) were employed as mobile phases. A 45-min linear gradient of 4–30% B was employed, and the flow rate was 0.80 mL/min. Under these conditions, we were able to resolve 5-methyl-2'-deoxycytidine (5-mdC) from other nucleosides. The global DNA cytosine methylation in cells was quantified based on the peak areas of 5-mdC and 2'-deoxycytidine (dC) with the consideration of the extinction coefficients of the two nucleosides at 260 nm (5020 and 7250 L mol⁻¹ cm⁻¹ for 5-mdC and dC, respectively).

Real-time PCR

Quantitative real-time PCR was used to quantify the mRNA expression of DNMT1. The primers were 5'-AGGGAAAAGGGAAGGGCAAG-3' (forward) and 5'-AGAAAACACATCCAGGGTCCG-3' (reverse). Untreated cells were used as control. SYBER Green real-time PCR was performed using 1 μ g of total RNA extracted with RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's recommended procedures. Data were analyzed using the comparative cycle threshold (Ct) method ($\Delta\Delta C_t$) where the GAPDH transcript level was used to normalize differences in sample loading and preparation (12). The experiment was biologically triplicated and the results represented the n-fold difference of transcript levels between different samples.

Western Blotting Analysis

Western blotting analysis was performed with the use of whole-cell lysates. Jurkat-T cells were washed with PBS and lysed via vortexing in an IP buffer (Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, supplemented with protease inhibitors before use). Protein concentrations were determined by using Bradford assay (Bio-Rad) with bovine serum albumin as standard. An equal amount of protein (60 μ g) from each sample was loaded onto 8% SDS-PAGE gels and separated using Tris-glycine buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3). Transferring to Protran nitrocellulose membrane (Whatman, Piscataway, NJ) was carried out by electroblotting in transfer buffer (10 mM NaHCO₃, 3 mM Na₂CO₃, pH 9.9, 20% methanol and 0.1% SDS) at 30 V for 12 h. Antibodies that specifically recognize human DNMT1 (New England Biolabs, Ipswich, MA), DNMT1-K142me (13), DNMT3a (Abcam, San Francisco, CA), DNMT3b (Abcam), human LSD1 (Cell Signaling, Danvers, MA), Set7 (Cell Signaling) and human β -actin (Abcam, Cambridge, MA) were used at 1:5000, 1:1000, 1:10000, 1:20000, 1:5000, 1:5000, and 1:10000 dilution, respectively. Horseradish peroxidase-conjugated secondary goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit antibodies (Abcam) were used at a 1:10000 dilution.

Results and Discussion

To assess precisely the global cytosine methylation in genomic DNA, we developed a straightforward and cost-effective HPLC-UV method to quantify the content of 5-methyl-2'-deoxycytidine (5-mdC) in genomic DNA. Figure 1A depicts the HPLC trace for the separation of nucleoside mixture produced from the digestion of genomic DNA isolated from Jurkat-T cells. In this context, we employed an acidic mobile phase (pH 4.0) to achieve a better separation between 5-mdC and residual guanosine arising from the small quantities of RNA present in the isolated genomic DNA. When comparing the HPLC trace of Figure

1A with the previously reported trace (14), the new mobile phase facilitated efficient separation of 5-mdC from other nucleosides (guanosine in particular), which afforded accurate quantification of the level of global cytosine methylation.

Chemotherapy is a key part of a patient's cancer treatment, which raises the question about whether chemotherapy can act as an external factor affecting cytosine methylation in tumor cells. Exposure of Jurkat-T cells to a variety of clinically used cancer chemotherapeutic agents affects the global DNA methylation levels in these cells (Figure 1B). In this regard, we observed apparent global DNA hypermethylation in Jurkat-T cells upon treatment with 10 μ M cyclophosphamide or arabinose C. On the other hand, treatment with 10 μ M doxorubicin or 3 μ M cisplatin led to obvious DNA hypomethylation, while BCNU and etoposide did not exert any appreciable effect on cytosine methylation. Among the six chemotherapeutic agents tested, cyclophosphamide induced the most pronounced change in cytosine methylation level in Jurkat-T cells. Therefore, we selected this drug for further investigation. Viewing that prolonged drug treatment is often required for cancer chemotherapy, we next asked if the cyclophosphamide-induced DNA hypermethylation could be maintained upon long-term drug administration. To this end, we treated Jurkat-T cells with 10 μ M cyclophosphamide for up to 10 days, harvested cells at different time points, and measured global cytosine methylation level in these cells (Figure 1C). Interestingly we found that cytosine methylation reached the highest level after 4 days of treatment, which resulted in an increase of the level of cytosine methylation by 17.3%, and the methylation level dropped subsequently to a similar level as untreated cells after 8 days (Figure 1C).

Viewing that DNMT1 is the major maintenance DNA methyltransferase in mammalian cells, we examined, by using Western blotting, whether the drug-induced perturbation in cytosine methylation was triggered by the altered expression of DNMT1 protein. Our results demonstrated that significant upregulation of DNMT1 protein indeed occurred after 4 days of cyclophosphamide exposure and the level of this protein returned to normal level after the cells were treated with the drug for 8 days (Figure 2A), which parallels the alteration in global DNA cytosine methylation level (Figure 1C). The levels of DNMT3a and DNMT3b, however, did not display significant changes upon cyclophosphamide treatment (Figure S2). These results strongly suggest that cyclophosphamide can induce the change in DNA methylation by perturbing the expression level of DNMT1 protein.

To explore the origin of cyclophosphamide-induced alteration in DNMT1 protein level, we next examined whether the drug perturbed DNMT1 expression through transcriptional regulation. It turned out that the mRNA level of DNMT1, as revealed by real-time PCR analysis, did not increase upon cyclophosphamide treatment (Figure S3); thus, cyclophosphamide altered DNMT1 protein level in Jurkat-T cells through a post-transcriptional mechanism.

Recent studies revealed that the stability of endogenous DNMT1 was regulated by its lysine methylation through histone lysine methyltransferase Set7 (13, 15) and histone lysine-specific demethylase 1 (LSD1) (15), which uncovered a mechanistic link between DNA and histone methylation systems. The methylated DNMT1 is susceptible to degradation via the ubiquitin-proteasome pathway (13). In addition, this mechanism is also at play in the 6-thioguanine-induced global cytosine demethylation in leukemia cells (10). Thus, we next asked whether lysine methylation also plays a role in cyclophosphamide-induced change in DNMT1 protein level. To this end, we investigated whether the drug could induce the alteration in the protein level of Set7 or LSD1. It turned out that treatment with cyclophosphamide led to a time-dependent increase in LSD1 protein level with up to 4 days of treatment and its level attenuated subsequently to that of untreated cells after 8 days of

treatment (Figure 2B and Figure S4A). On the other hand, no substantial perturbation in Set7 expression was found (Figure 2C and Figure S4B). These results further suggested that cyclophosphamide could induce the alteration in the level of LSD1 protein, which may result in diminished lysine methylation in DNMT1 thereby minimizing its degradation via the proteasomal pathway. To explore this pathway further, we assessed the level of K142 methylation in DNMT1 during the course of cyclophosphamide treatment. We initially attempted to isolate DNMT1 from Jurkat-T cells by immunoprecipitation with anti-DNMT1 antibody and characterize the methylation of K142 in this protein using LC-MS/MS; unfortunately we were not able to obtain adequate amount of protein for the mass spectrometric experiment. However, our Western blot result using a previously reported antibody (13) showed that there is a slight increase in K142 methylation level after 1 and 4 days of cyclophosphamide treatment (Figure 2D and Figure S4C). At first glance, this appears to be inconsistent with what we predict from the observed change in LSD1 and Set7 levels. However, considering the marked elevation in the level of DNMT1 protein after 1 and 4 days of drug treatment, we may conclude that the fraction of DNMT1 carrying the K142 methylation decreased upon 1 and 4 days of drug exposure.

Conclusions

Perturbation in epigenetic pathways is known to be associated with cancer development (4). A previous study revealed that some widely prescribed chemotherapeutic agents could perturb cytosine methylation in tumor cells at toxic concentrations (11). We found that cyclophosphamide, when administered at a more therapeutically relevant concentration (10 μ M), could induce a transient increase in cytosine methylation level by upregulating temporarily the expression of LSD1 and DNMT1 proteins in Jurkat-T cells. The increase and subsequent decrease in global DNA cytosine methylation level may reflect cells' adaptive response to this widely used anticancer agent, which may bear important implications in the development of resistance to this drug during cancer chemotherapy. In this vein, drug efflux from cancer cells mediated by ATP-binding cassette (ABC) transporters is one of the most common mechanisms for cancer cells to develop resistance toward anti-neoplastic agents (16). It can be envisaged that the treatment with cyclophosphamide may result in elevated expression of some ABC transporters and increased drug efflux. This may account for the returning of LSD1, DNMT1, and global cytosine methylation levels to those of untreated cells 4 days after the drug treatment. However, the molecular events underlying the drug-induced perturbation in LSD1 protein level warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (R01 DK082779).

References

1. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* 2005; 74:481–514. [PubMed: 15952895]
2. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 1999; 99:247–257. [PubMed: 10555141]

3. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*. 1992; 69:915–926. [PubMed: 1606615]
4. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010; 31:27–36. [PubMed: 19752007]
5. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N. Eng. J. Med*. 2003; 349:2042–2054.
6. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res*. 2000; 60:892–895. [PubMed: 10706101]
7. Rosas SLB, Koch W, Carvalho MDD, Wu L, Califano J, Westra W, Jen J, Sidransky D. Promoter hypermethylation patterns of p16, O-6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer Res*. 2001; 61:939–942. [PubMed: 11221887]
8. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM, Ellerton J, Larson RA, Schiffer CA, Holland JF. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: A study of the cancer and leukemia group B. *J. Clin. Oncol*. 2002; 20:2429–2440. [PubMed: 12011120]
9. Kantarjian H, Issa JPI, Rosenfeld CS, Bennett JM, Albitar M, DiPersio J, Klimek V, Slack J, de Castro C, Ravandi F, Helmer R, Shen LL, Nimer SD, Leavitt R, Raza A, Saba H. Decitabine improves patient outcomes in myelodysplastic syndromes - Results of a Phase III randomized study. *Cancer*. 2006; 106:1794–1803. [PubMed: 16532500]
10. Yuan B, Zhang J, Wang H, Xiong L, Cai Q, Wang T, Jacobsen S, Pradhan S, Wang Y. 6-Thioguanine reactivates epigenetically silenced genes in acute lymphoblastic leukemia cells by facilitating proteasome-mediated degradation of DNMT1. *Cancer Res*. 2011; 71:1904–1911. [PubMed: 21239472]
11. Nyce J. Drug-induced DNA hypermethylation and drug-resistance in human-tumors. *Cancer Res*. 1989; 49:5829–5836. [PubMed: 2790794]
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
13. Esteve PO, Chin HG, Benner J, Feehery GR, Samaranayake M, Horwitz GA, Jacobsen SE, Pradhan S. Regulation of DNMT1 stability through SET7-mediated lysine methylation in mammalian cells. *Proc. Natl. Acad. Sci. U S A*. 2009; 106:5076–5081. [PubMed: 19282482]
14. Wang HX, Wang YS. 6-Thioguanine perturbs cytosine methylation at the CpG dinucleotide site by DNA methyltransferases in vitro and acts as a DNA demethylating agent in vivo. *Biochemistry*. 2009; 48:2290–2299. [PubMed: 19236003]
15. Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, Gaudet F, Li E, Chen T. The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. *Nat. Genet*. 2009; 41:125–129. [PubMed: 19098913]
16. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat. Rev. Drug Discov*. 2006; 5:219–234. [PubMed: 16518375]

Figure 1.

(A) The HPLC trace for the separation of nucleoside mixture from the enzymatic digestion of genomic DNA isolated from untreated Jurkat-T cells. Shown in the insets are the tandem mass spectrum of the $[M+H]^+$ ion of 5-mdC (m/z 242) and the expanded chromatogram to visualize better the 5-mdC peak. "G" represents guanosine. (B) The level of cytosine methylation in Jurkat-T cells that were exposed to the indicated concentrations of anticancer drugs for 24 hr. Results are reported as the percentages of cytosine being methylated (see Experimental Procedures). (C) Cyclophosphamide-induced alteration of 5-mdC levels in drug-exposed Jurkat-T cells was monitored for up to 10 days. The culture medium was changed and freshly prepared drug was added daily at a final concentration of 10 μ M.

Results shown in (B) and (C) are the means and standard deviations of data from three independent drug exposure and HPLC measurements.

Figure 2. Western blot results for DNMT1 (A), LSD1 (B), and Set7 (C) and DNMT1 with monomethylation on K142 (D) in the whole cell lysate of Jurkat-T cells treated with 10 μ M cyclophosphamide for the indicated periods of time. The culture medium was changed and freshly prepared drug was added daily at a final concentration of 10 μ M. β -actin was used a loading control.