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The Effect of 6-Thioguanine on Proliferation, Viability and Expression of the Genes DNMT 3A, DNMT 3B and HDAC3 in Lymphoid Cancer Cell Line Nalm6

Tohid Rostamian MSC[1], Fatemeh Pourrajab PhD[1,*], Seyedhossein Hekmatimoghaddam MD[2,3,*]

1. Department of Biochemistry and Molecular Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
2. Hematology & Oncology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
3. Zoonotic Diseases Research Center, Department of Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
*Corresponding author: Dr Fatemeh Pourrajab & Seyedhossein Hekmatimoghaddam, Hematology & Oncology Research Center, Shahid Sadoughi Hospital, Safaiyeh, Yazd, Iran. E-mail: shhekmati2002@yahoo.com, ORCID: 0000-0001-9859-375; mina_poorrajab@yahoo.com, ORCID: 0000-0002-1724-5392.

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Abstract

Background: 6-thioguanine (6-TG) is one of the thiopurine drugs with successful use in oncology, especially for acute lymphoblastic leukemia (ALL). 6-TG is proposed to act as an epigenetic drug affecting DNA methylation. The aim of this study was to clarify the effect of 6-TG on the proliferation, viability and expression of genes coding for the enzymes DNA methyltransferase 3A and DNA methyltransferase 3B (DNMTs) as well as histone deacetylase 3 (HDAC3) in the human B cell-ALL cell line Nalm6.

Materials and Methods: In this experimental study, Nalm6 cells and also normal peripheral blood mononuclear cells (PBMCs) were grown in RPMI 1640 medium containing 10% fetal bovine serum. They were then treated with 6-TG at their exponential growth phase. Cell viability was monitored using the Cell Counting Kit-8 assay with an enzyme-linked immunosorbent assay (ELISA) reader. The expressions of the above-mentioned 3 genes were quantified using real-time PCR.

Results: 6-TG could inhibit the proliferation of Nalm6 cells and decrease their viability. In Nalm6 cells, as compared to normal PBMCs, 6-TG significantly decreased HDAC3 ($p = 0.008$) as well as DNMT3B ($p = 0.003$) gene expressions, but increased the expression of DNMT3A gene ($p = 0.02$) after normalization to GAPDH, as the housekeeping gene.

Conclusion: These findings suggested that the altered expression of DNMT3A, DNMT3B and HDAC3 genes was responsible for at least part of the antitumoral properties of 6-TG, providing an insight into mechanism of its action as an epigenetic drug.

Keywords: DNA methyltransferase, Histone deacetylase, Leukemia, Thioguanine, Thiopurine

Introduction

Epigenetic changes are heritable and reversible changes that alter the gene expression without involving nucleotide sequence of genes. Among others, histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) are the main enzymes responsible for modification of genotype expression into a particular phenotype. These changes include DNA methylation, histone modification, and regulatory patterns for micro RNAs in the process of tumorigenesis. They have important roles in the development of various cancers such as acute lymphoblastic leukemia (ALL) (1,2).

One of the major epigenetic modifications that play important roles in embryonic development, gene regulation, cell differentiation, and genomic imprinting is methylation of DNA at the C5 of cytosine at CpG dinucleotide (3,4). Aberrant methylation within CpG islands in the genome leads to genomic instability and subsequently development of many diseases, including cancer (5,6). Promoter

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CpG methylation generally correlates with gene silencing, including in tumor-suppressor genes (7,8). Therefore, it seems that demethylation of promoter cytosine residues and the resultant reactivation of silenced genes in cancer cells could be approached in cancer therapy. In mammalian cells, DNA methylation is established and maintained by a family of DNMTs, including DNMT1, DNMT3A and DNMT3B (9). DNMT1 activity causes suppression of genes, resulting in activation of multiplicative stimulant genes. In contrast, DNMT3A activity plays a role in the extinction of multiplicative stimulatory genes and the activation of multiple suppressor genes. Increasing or decreasing the expression of these enzymes can play a role, in particular, in the development of ALL or progression of the disease (10).

DNMTs are upregulated in several human cancers (11,12). Earlier studies showed that the regulatory regions of tumor-suppressor genes were hypermethylated in tumors, as evident from methylome sequencing of promoter areas. Therefore, DNMTs have been suggested as a target for anticancer therapy (13,14). Thiopurine drugs are widely used for their anticancer, antimicrobial, and immunosuppressive effects, with considerable success in clinical practice, especially for the treatment of ALL (15-18). The final active metabolite of all thiopurine prodrugs is 6-TG. It is suggested that 6-TG exerts its cytotoxic effect by incorporation into DNA. It is then methylated by S-adenosyl-L-methionine to render 6-methylthioguanine, leading to misincorporation of deoxythymidine monophosphate (dTMP) during DNA replication (18).

In addition to the melt transfer activity, DNMTs play a role in modulating and rearranging the chromatin structure and call for proteins involved in the expression of genes. For example, DNMTs call for HDACs to promoter regions and interact directly with them. HDACs proteins play a role in silencing the gene (19).

Up to now, at least 18 members of the HDAC family have been identified in mammalian cells, being assigned into four classes I, II (IIA and IIB), III, and IV. Class I includes HDAC1,2,3,8, Class IIA includes HDAC4,5,7,9, Class IIB includes HDAC6,10, and Class IV includes only HDAC11. In studies of animal models or cell lines, it was suggested that HDACs 1,2,3,8 were involved in proliferation, HDACs 4,6,7,10 in angiogenesis, HDAC6 in migration, HDAC1.2 in inhibiting apoptosis, HDACs 3,4,5,8 in decomposition, and HDAC1 in chemotherapy resistance, indicating that members of this family play a major role in the development of cancer (20).

The amount of histone acetylation is determined by the balance between the activity of the histone acetyltransferase (HAT, which induces the acetylation of histones and reduces their interactions with DNA) and HDAC enzyme (which removes the acetyl group from the lysine amino acids in the histones). Their alteration in a number of cancers, especially ALL, has been reported to be the result of somatic mutation, alleviation, or reduction of its expression (21). The possibility of creating therapeutic goals and, in particular, new drugs (using their inhibitors) acting on HDACs has been proposed for the treatment of cancers (22).

Thiopurines which include 6-TG, 6-mercaptopurine, and azathioprine, are known to be effective in treating ALL (23,24). However, their mechanism of action has not been clearly identified. According to some studies, these drugs have an epigenetic effect. For example, 6-TG decreases DNMT1 levels and clarifies the expression of exogenous genes in cancer cells (23). The cells are differentiated through the expression of DNMT1, DNMT3A and DNMT3B during development. However, the type and amount of these enzymes differ in the early stages of differentiation versus the

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final stage of differentiation. It is also reported that when the cell encounters DNA- damaging drugs, it will increase the ability to express DNMT1 and DNMT3A (25).

Since the thiopurine drug 6-TG is used extensively in the treatment of acute leukemias (26-28), recent studies have shown that HDAC3 can be a potential target for the development of new therapeutic agents (29), and because of uncertainties about mode of action of 6-TG, it was decided to study its effect on proliferation, viability and epigenetic genes of DNMT3A, DNMT3B, and HDAC3 in the ALL cell line Nalm6.

Materials and Methods

Reagents and cell line:

This lab trial experiment was conducted in Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and approved by the ethics committee (code IR.SSU.MEDICINE.REC.1396.133). The human B-cell precursor leukemia cell line Nalm6 was provided from Institute Pasture, Tehran, Iran. As healthy control group, peripheral blood mononuclear cells (PBMCs) from a pool of 10 healthy asymptomatic children (< 12 years old) referred to Yazd Central Medical Laboratory were taken by Ficoll gradient method. All cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sinaclon, Iran), penicillin (50 units/mL, Gibco, USA), and streptomycin (50 µg/mL, Gibco, USA) at 37 °C in a 95% humidified atmosphere with 5% CO_2. The culture flasks were diluted at a ratio of 1:3 every one to two days. 6-TG (Sigma, USA) was dissolved at 0.034 M in dimethyl sulfoxide (DMSO, Sigma, USA) as a stock solution, with dilution in serum-free RPMI 1640 medium just before use. The maximum final concentration of DMSO in medium was < 0.02%.

Cell growth and cytotoxicity assay:

Cell viability was measured with the highly water-soluble reagent 2-(2-methoxy-4-nitrophenyl)-3-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (cell counting kit-8, CCK-8) assay. Briefly, exponentially-growing Nalm6 cells at 1.25×10^4 cells/well were treated with 0.8 ng (1/10 of the lethal concentration, 5 µM) of 6-TG in each well or without 6-TG (in the control wells) in a 96-well cell culture plate to a total volume of 100 µL per well. After the incubation of cells for 24 and 48 h, 10 µL of CCK-8 solution (Beyotime, China) was added to each well and incubation continued for another 4 h at 37 °C. The relative cell viability was determined by scanning with an enzyme-linked immunosorbent assay (ELISA) reader (Awareness Technology Inc., USA) with a 450 nm filter and calculated by CCK-8 assay.

RNA Isolation from the cells treated with 6-TG:

Total RNA from 6-TG-treated cells was extracted according to the RNeasy kit (Sinaclon, Iran). The quantity and quality of RNA content was checked using Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Sample yield, quality, and purity were determined through absorbance ratios and concentrations.

Reverse transcription and cDNA synthesis:

To evaluate the primers by RT-PCR and measure the expression of genes by real-time PCR (quantitative PCR, QPCR), the isolated total RNA was transcribed into cDNA by the use of the High Capacity cDNA Reverse Transcription Kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA). Briefly, one µg of RNA was mixed with 1 µL of the random hexamer primer followed by addition of nuclease-free water (Qiagen, Germany) up to 12 µL, according to the kit instructions. After incubation of the tubes in a thermocycler at 65 ˚C for 5 minutes the tubes were placed on ice (4 ˚C) and the other reagents were added. The resulting first strand cDNA was then amplified with

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the following program: 5 minutes at 25 ˚C, 60 minutes at 42 ˚C, and 5 minutes at 70 ˚C.

QPCR:

Absolute quantification and relative quantification are the two most commonly used methods to analyze data from QPCR experiments. The $2^{-\Delta\Delta ct}$ method is a useful simple way to analyze the relative changes in gene expression from QPCR. To quantify the mRNA levels of target genes, QPCR was done using cDNA, forward and reverse primers, distilled water and EvaGreen qPCR Mastermix 5× (as a mixture of dNTPs, Hotstart Taq polymerase [HOT FIREPol®, made by Solis BioDyne, Tartu, Estonia], $MgCl_2$, fluorescent detection dye EvaGreen, reference dye, and proprietary buffer components), according to the kit instructions. Unlike SYBR® Green I, EvaGreen® dye is cell membrane impermeable, and therefore cannot bind DNA in living cells. It has much less PCR inhibition, is extremely stable dye, has been shown to be nonmutagenic and noncytotoxic, and imparts brilliant green fluorescence to dsDNA. Relative expression of the target genes was performed by Step One Plus Real-time PCR (Applied Bio systems, USA) in duplicate to a final volume of 20 µL using pre-set cycling parameters (10 min at 95 °C; 15 s at 95 °C; 20 s at 60 °C with the latter two steps repeated for 35 times), and was then quantified by the ΔΔCT method. The expression of Parp1 mRNAs was normalized to GAPDH (Applied Biosystems; assay ID: Mm99999915_g1) as the endogenous reference in the corresponding samples, and relative to the untreated control cells. The primer sequences used in QPCR are listed in Table I.

Statistical analysis:

Each experiment was performed in triplicate. The data were presented using mean ± SD, unless stated otherwise. The statistical difference between groups was determined by Student's t- test, one way ANOVA, and Tukey's studentized range test, where appropriate. Differences between groups were considered statistically different at $P < 0.05$**.**

Results

Inhibitory effect of 6-TG on the proliferation of lymphoblast Nalm6 cells

To determine the effect of 6-TG on the growth of B-ALL cells, Nalm6 cells were treated with 6-TG followed by manual cell counting after 24 h and 48 h. Compared with the control group, the cell density of the group treated with 5.0 µM 6-TG was increased only a little from 24 h to 48 h, indicating that 6-TG significantly inhibited the growth of Nalm6 cells. CCK-8 assays showed that the viability of Nalm6 cells, exposed to different concentrations of 6-TG (1.25, 2.5, and 5.0 µM), was decreased from 82% to 54% after 24 h and from 80% to 42% after 48 h, suggesting that 6-TG inhibited the proliferation of Nalm6 cells.

Decreased expression of HDAC3 gene in lymphoid cell line Nalm6 treated with 6-TG

The expression of HDAC3 gene in the Nalm6 lymphoid group and normal human blood cells was measured before and after treatment with 6-TG. Significant decrease in Nalm6 cells 24 h after treatment was found, which approached the level of gene expression in the normal group ($p = 0.008$) ($p = 0.05$) (about 32% reduction, at 95% confidence level) (Figure 1). These results revealed that 6-TG may directly cause cell-cycle arrest in the G0/G1 phase, similar to the effects induced by lycorine (30).

Down-regulation of DNMT3B by 6-TG

The expression of DNMT3B gene in Nalm6 lymphoid and normal human blood cells was measured before and after treatment with 6-TG, which showed a significant ($p = 0.003$) reduction after 24 hours (by about 75.8% at 95% confidence level). In addition, it approached the level of gene expression in the control group (Figure 2).

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After analyzing with one-way ANOVA or t-test, where appropriate, values were considered significantly different with p = 0.008 (*).

Increased expression of DNMT3A in lymphoid cell line Nalm6 treated with 6-TG

The expression of DNMT3A gene in Nalm6 cells after 24 hr showed a significant (p = 0.02 at 95% confidence level) increase (by about 8 times more than normal human blood mononuclear cells) (Figure 3).

After analyzing with one-way ANOVA or t-test, where appropriate, values were considered significantly different with p = 0.008 (*).

The relative fold changes in the expression of all 3 genes before versus after treatment with 6-TG is shown in Figure 4.

After analyzing with one-way ANOVA or t-test, where appropriate, values were considered significantly different with p = 0.008 (*).

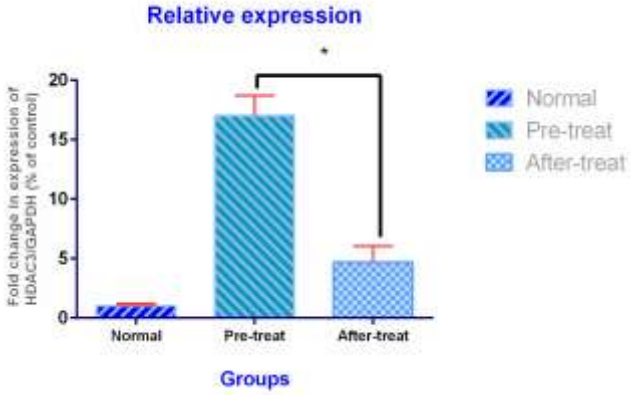

Figure 1. The relative fold change in the expression of HDAC3 in the cell line Nalm6 and in healthy control group (as calibrator) after normalization to GAPDH which is expressed constantly in every cell type. After analyzing with one-way ANOVA or t-test, where appropriate, values were considered significantly different with p = 0.008 (*).

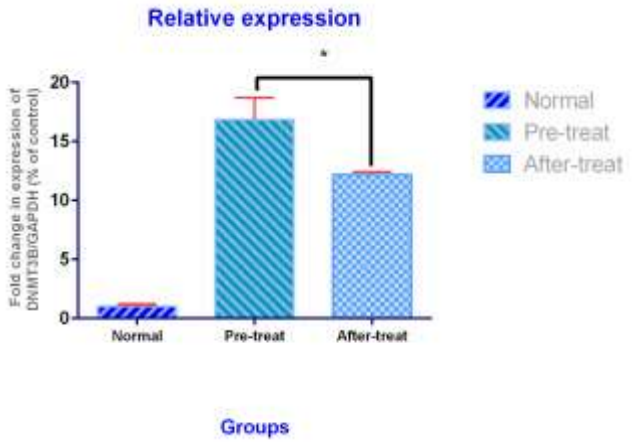

Figure 2. The relative fold change in the expression of DNMT3B in the cell line Nalm6 and in healthy control group (as calibrator) after normalization to GAPDH, which was expressed constantly in every cell type.

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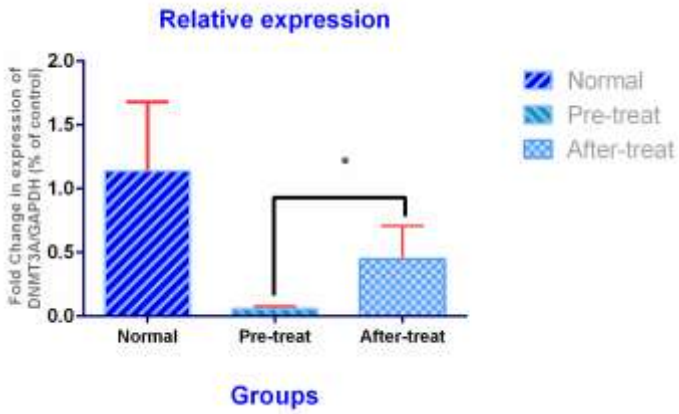

Figure 3. The relative fold change in the expression of DNMT3A in the cell line Nalm6 and in healthy control group (as calibrator) after normalization to GAPDH, which was expressed constantly in every cell type.

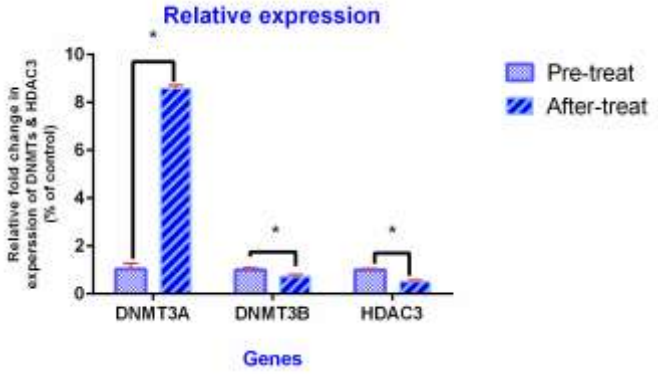

Figure 4. The relative fold change in expression of DNMT3A, DNMT3B, and HDAC3 in the cell line Nalm6 and in healthy control group (as calibrator) after normalization to GAPDH, which was expressed constantly in every cell type.

Table I: Primers used in the study

Gene	Primer Sequence	Product Size, bp
DNMT3A	F: 5'- CCCAAGGTCAAGGAGATTA-3' R: 5'-CAGATGTCCTCAATGTTCCG-3'	90
DNMT3B	F: 5'-CCTTCTTCACCAGTGACACG -3' R: 5'-CCATGACAGGACTCGAATGG-3'	98
HDAC3	F: 5'-CCAAGACCGTGGCCTATTT-3' R: 5'-AATGCAGGACCAGGCTATG-3'	111
GAPDH	F: 5'-GAGCCACATCGCTCTGACAC-3' R: 5'-CATGTAGTTGAGGTCAATGAAGG-3'	157

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Discussion

The present experimental study was designed to determine the effect of 6-TG treatment on the acute B-lymphoblastic leukemia cell line Nalm6. The expression of three genes, the DNMT3A, DNMT3B, and HDAC3 was measured. The role of epigenetic enzymes in human development and cancers has been under study since more than 2 decades before (31). It is said that more than half of all cancer types show mutations in the genes for epigenetic enzymes, including DNMTs and HDACs. For example, the genes encoding isocitrate dehydrogenase in gliomas and acute myeloid leukaemia (AML) reveal mutations which inhibit the activity of histone demethylases and DNA demethylases, leading to altered DNA and histone methylation patterns (32). The evidence suggests that DNA methylation plays a very important role in leukemia, and the amount of DNMT expression can play an important role in the progression of the disease.

Our study found a significant increase in the expression of DNMT3A and significant decrease in the expression of DNMT3B and HDAC3 following treatment with 6-TG. Due to known high frequency of mutations of DNMTs in leukemias (33), a number of clinical trials or in vitro studies are undertaken or ongoing, using DNMT inhibitors, especially in AML. For example, according to a study on DNMT3A in the cell line Nalm6 with the results quite similar to the current study, treatment with thiopurines increased the DNA methylation significantly from 67% to 72% (34).

On the other hand, according to studies by Sayin et al., in 2010, it was deduced that the activity of DNMT1 and DNMT3B was abnormally increased in ALL patients, in line with finding in this study. According to aforementioned study, the transcription of DNMT3B gene is associated with increased activity of the proliferating cell nuclear antigen (PCNA, a factor involved in DNA similarity and cell proliferation) that can be effective in the proliferation of cells (35). So, the expression of these genes can be associated with the progression of ALL. DNMT3B plays an important role in the development and differentiation of hematopoietic stem cells, and in some malignancies. The role of mutations in the deactivation and disruption of the enzyme in ALL and AML has been studied in a large number of patients (36).

HDACs generally induce differentiation in the tumor cells, inhibit cellular oscillation in the G0/G1 or G2/M stage, and activate apoptotic genes, which depend on the type of cell (30). In 2001, it is reported that HDACs play a role in regulating the expression of genes. Specifically, 3 HDACs can suppress receptors for retinoic acid and thyroid receptors. They also affect other biological activities and their regulation, such as differentiation, proliferation, apoptosis, and cell division (23). The results of this study showed that 6-TG reduced the expression of HDAC3 in the lymphoid cell line, which is close to its expression level in normal cells. The expression of DNMT3A and DNMT3B genes in Nalm6 cell lines, as well as in the control group, was approaching the normal level after treatment with 6-TG. This altered expression could potentially and theoretically be associated with cell cycle arrest in G0/G1 phase.

It is obvious that mechanisms other than effect on gene expression change in DNMTs and HDACs could be involved in AML response to thiopurines, and mutations in these genes must be considered, too (37). Studying those mutations and some tumor suppressor genes which might be affected in AML could be mentioned as one of our limitations in this study.

Conclusion

Findings of this study suggested that the altered expression of DNMT3A, DNMT3B, and HDAC3 genes was

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responsible for at least part of the antitumoral properties of 6-TG, providing an insight into mechanism of its action as an epigenetic drug. The findings of the present study are intended to increase our insight into the mechanism of antineoplastic activity of 6-TG. There certainly would be a need for further research toward development of more drugs targeting epigenetic factors.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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