The Impact of 6-Thioguanine on Epigenetics of Acute Myeloid Leukemia

Tohid Rostamian, Seyedhossein Hekmatimoghaddam, Fatemeh Pourrajab

1) International Campus, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
2) Hematology and Oncology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
3) Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
4) Nutrition and Food Security Research Centre, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
5) Department of Biochemistry and Molecular Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Abstract
The drug 6-thioguanine (6-TG) is one of the thiopurines successfully used in oncology, especially for acute myeloid leukemia (AML). It 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) and histone deacetylase 7 (HDAC7) in the human promyelocytic AML cell line HL60.

In this experimental study, HL60 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 4 genes were quantified using real-time PCR.

6-TG could inhibit the proliferation of HL60 cells and decrease their viability. In HL60 cells, as compared to normal PBMCs, 6-TG significantly decreased HDAC3 (p = 0.0034) as well as DNMT3B (p = 0.03) and HDAC7 (p = 0.0031) gene expressions, but increased the expression of DNMT3A gene (p = 0.16) after normalization to GAPDH as the housekeeping gene.

These findings suggest that the altered expression of DNMT3A, DNMT3B, HDAC3 and HDAC7 genes is responsible for at least part of the antitumor properties of 6-TG, providing an insight into the mechanism of its action as an epigenetic drug.

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

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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 acute promyelocytic leukemia cell line HL60 was provided from Institute Pasteure, Tehran, Iran. As the control group, peripheral blood mononuclear cells (PBMCs) from a pool of 10 healthy asymptomatic children referred to Yazd Central Medical Laboratory were obtained by Ficoll gradient method. All cells were grown in RPMI 1640 medium (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (Sinaclon, Mashhad, Iran), penicillin (50 units/mL, Gibco, USA), and streptomycin (50 μg/mL, Gibco, USA) at 37°C in a 95% humidified atmosphere with 5% CO₂. The culture flasks were diluted at a ratio of 1:3 every one to two days. 6-TG (Sigma, St Louis, USA) was dissolved at 0.034 M in dimethyl sulfoxide (DMSO) (Sigma, St Louis, 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 watersoluble reagent 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (cell counting kit-8, CCK-8) assay (Beyotime, China). Briefly, exponentially growing HL60 cells at 1.25 × 10⁴ 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, 48 and 72 h, 10 μL of CCK-8 solution 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., Westport, USA) with a 450 nm filter and calculated by CCK-8 assay instructions.

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

cDNA Synthesis and Reverse Transcription Polymerase Chain Reaction (RT-PCR)
To evaluate the primers by RT-PCR and measure the expression of genes by 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 Fisher Scientific, Waltham, USA). Briefly, one μg of RNA was mixed with 1 μL of the random hexamer primer followed by addition of nuclease-free water (Qiagen, Venlo, 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 the following program: 60 minutes at 42°C, and 5 minutes at 70°C.

QPCR Target Determination
Absolute quantification and relative quantification are the two most commonly used methods to analyze data from QPCR experiments. The 2⁻ΔΔct 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®, Solis BioDyne, Tartu, Estonia], MgCl₂, 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, 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 Biosystems, Waltham, USA) in duplicate to a final volume of 20 μL using pre-set cycling parameters: Initial hold at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 20 s. A final hold at 72°C for 600 s ensured maximum extension. For the melting analysis, the following protocol was applied: Ramp from 65°C to 95°C, hold for 90 s on the first step, and hold for 5 s on next steps. It was then quantified by the 2⁻ΔΔct formula. The expression of 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 1.

Statistical Analysis
All statistics were performed by the Prism GraphPad V8. Sensitivity and specificity calculations were performed using receiver operator characteristic curves. Unpaired samples were compared using the Mann-Whitney test and paired analyses were performed using t-test, one way ANOVA, and Tukey’s studentized range
test, whichever appropriate. Differences between groups were considered statistically different at $P < 0.05$. Each experiment was performed in triplicate. The data were presented using mean ± SD.

**Results**

**Inhibitory Effect of 6-TG on the Proliferation of Myeloid HL60 Cells**

Compared with the control group, the density of the HL60 cell group treated with 5.0 μM 6-TG was increased only a little from 24 h to 48 h and 72 h, indicating that 6-TG inhibited the growth of HL60 cells. CCK-8 assays showed that the viability of HL60 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 to 42% after 48 h, suggesting that 6-TG inhibited the proliferation of HL60 cells.

**Decreased Expression of the HDAC3 Gene**

Significant decrease in the expression of HDAC3 gene in HL60 cells after treatment with 6-TG was found ($p = 0.0034$ at 95% confidence level) which approached the level of gene expression in the normal group (Fig. 1).

**Decreased Expression of the HDAC7 Gene**

The expression of HDAC7 gene in the HL60 lymphoid myeloid group after treatment with 6-TG showed significant decrease ($p = 0.0031$ at 95% confidence level), which is closer to the level of gene expression in the normal group (Fig. 2).

**Reduced Expression of the DNMT3B Gene**

The expression of DNMT3B gene in HL60 myeloid cells was decreased after treatment with 6-TG (Fig. 3). Values were considered significantly different with $p = 0.030$ at 95% confidence level.

**Increased Expression of the DNMT3A Gene**

The expression of DNMT3A gene in HL60 myeloid cells showed a significant (at 95% confidence level) increase (Fig. 4). After analysis with one-way ANOVA, table 1. Primers used in the study

| Gene   | Primer Sequence                                      | Product size, bp |
|--------|------------------------------------------------------|------------------|
| DNMT3A | F: 5'-CCCAAGTGCAAGGAGATTAC3'-R: 5'-CGATGTCTTATCGGATCCG3'- | 90               |
| DNMT3B | F: 5'-CCCTCCACACGATGACG3'-R: 5'-CCATGACAGGACCTGGAATGG3'- | 98               |
| HDAC7  | F: 5'-GGGACAACGTGATTCATGTC3'-R: 5'-AGTACGCTTACCATCTAATG3'- | 111              |
| HDAC3  | F: 5'-CCAGACGGCCGTGGCTATTTCTGCC3'-R: 5'-AATGCGACGGGATCGG3'- | 125              |
| GAPDH  | F: 5'-GAGCCACATCGCTCTGACAC3'-R: 5'-CATGTAGTTGGATGGAATGG3'- | 157              |

Fig. 1 The relative fold change in the expression of HDAC3 in the cell line HL60 and the healthy control group (as calibrator) after normalization to GAPDH which is expressed constantly in every cell type. After analyzing with one-way ANOVA, the pre-treatment value was significantly different from post-treatment values with $p = 0.0034$ (*).

Fig. 2 The relative fold change in the expression of HDAC7 in the cell line HL60 and the healthy control group (as calibrator) after normalization to GAPDH which is expressed constantly in every cell type. Analysis with one-way ANOVA. The pre-treatment value was significantly different from post-treatment values with $p = 0.0031$ (*).
values were considered significantly different with \( p = 0.016 \).

The relative fold changes in the expression of all 4 genes before versus after treatment with 6-TG is shown in Fig. 5.

**Discussion**

This experimental study tried to assess the effect of 6-TG treatment on the myeloid leukemia cell line HL60. According to the analysis, the drug was able to inhibit the growth and proliferation of HL60 cells. DNMTs and HDACs are closely related to each other and involved in various human diseases especially cancer. These two enzymes have been widely recognized as antitumor targets for drug discovery. Besides, research has indicated that combination therapy consisting of DNMT and HDAC inhibitors exhibits therapeutic advantages\(^\text{11}\). 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. Recent studies have attempted to develop new small molecules targeting epigenetic modifying enzymes like DNMT, histone methyltransferases or HDACs with the aim of inducing differentiation in AML\(^\text{12}\).

Our study found a significant increase in the expression of DNMT3A and significant decrease in the expression of DNMT3B, HDAC3 and HDAC7 following treatment with 6-TG. Due to known high frequency of mutations of DNMT3A in AML, there may be both
increased and decreased function of the enzyme\textsuperscript{(13)}. DNMT3B plays an important role in the differentiation of hematopoietic stem cells, embryo development and in some malignancies. There have been conflicting results from studies on its role either as a tumor suppressor or accelerator of progression in AML\textsuperscript{(14,16)}. HDAC inhibitor-induced apoptosis in AML has been documented, and is consistent with our findings\textsuperscript{(17)}.

One of the limitations in our study was lack of assessment of gene mutations and some tumor suppressor genes which might be affected in AML.

**Conclusion**

Findings of this study suggest that altering the expression of DNMT3A, DNMT3B, HDAC3 and HDAC7 genes may be responsible for at least part of the antitumor properties of 6-TG, providing an insight into mechanism of its action as an epigenetic drug. There certainly would be a need for further research toward development of more drugs targeting epigenetic factors.

**Declaration of Interest:**

None of the authors has any conflict of interest to declare.

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