

Investigation of Decitabine Effects on HDAC3 and HDAC7 mRNA Expression in NALM-6 and HL-60 Cancer Cell Lines

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Abstract

Background: Decitabine is a potent anticancer hypomethylating agent and changes the gene expression through the gene's promoter demethylation and also independently from DNA demethylation. So, the present study was designed to distinguish whether Decitabine, in addition to inhibitory effects on DNA methyltransferase, can change HDAC3 and HDAC7 mRNA expression in NALM-6 and HL-60 cancer cell lines.

Methods: HL-60, NALM-6, and normal cells were cultured, and the Decitabine treatment dose was obtained (1 μ M) through the MTT assay. Finally, HDAC3 and HDAC7 mRNA expression were measured by Real-Time PCR in HL-60 and NALM-6 cancerous cells before and after treatment. Furthermore, HDAC3 and HDAC7 mRNA expression in untreated HL-60 and NALM-6 cancerous cells were compared to normal cells.

Results: Our results revealed that the expression of HDAC3 and HDAC7 in HL-60 and NALM-6 cells increases as compared to normal cells. After treatment of HL-60 and NALM-6 cells with Decitabine, HDAC3, and HDAC7 mRNA expression were decreased significantly.

Conclusions: Our data confirmed that the effects of Decitabine are not limited to direct hypomethylation of DNMTs, but it can indirectly affect other epigenetic factors, such as HDACs activity, through converging pathways.

Keywords: Decitabine, HDAC3, HDAC7, HL-60, NALM-6.

Introduction

Epigenetics changes mean gene function cannot be explained by changes in DNA sequences(1). These changes are inherited and do not affect the DNA nucleotide sequence. There are multiple types of epigenetic mechanisms, such as non-coding RNA regulation, DNA methylation, and histone modifications. Histone modifications mediated by specific histone regulatory enzymes alter gene expression (2, 3). There are five recognized types of histones, H1, H2A, H2B, H3, H4, and H5. Histone modifications are regulated by

multiple enzymes, including methyltransferases (HMTs), demethylase (HDM), acetyltransferase (HATs), and deacetylases (HDACs) (2, 4). HDACs are classified into four groups; HDACs group I, present in the nucleus, including HDAC 1, 2, 3, and 8. Group II HDACs, including HDAC 4, 5, 6, 7, 9, and 10, migrate between the nucleus and cytoplasm. HDACs Class I are often expressed ubiquitous, whereas class IIa HDACs are expressed in a limited number of various cells (5). HDACs do not correlate

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DNA directly; instead, they are believed to be recruited to particular promoters via their interaction with DNA sequence-specific transcription agents. Some studies confirmed that HDAC inhibitors (HDACi) could initiate apoptosis and arrest the cell cycle in acute lymphoblastic leukemia (ALL) cell cultures (6-8). Other studies proved that HDAC7 and HDAC9 mRNA overexpression was considered to be associated with bad prognostic agents and a higher risk of fatal outcomes (9). Moreover, it has been shown that HDAC7 may control the beginning of apoptosis. High expression of HDAC7 was also observed in pancreas adenocarcinomas (10, 11).

There are many epigenetic medications such as bromodomain and inhibitors, HAT inhibitors, protein methyltransferase inhibitors, histone methylation inhibitors, methylation inhibitors, and HDAC inhibitors. 2'-deoxycytidine-5-Aza or Decitabine is an epigenetic medicine, a decisive hypomethylating factor, and usually used to demethylate some gene's promoter (12, 13). Various scientific studies determine that Decitabine changes the gene expression through demethylation of the gene's promoter and independently from DNA demethylation (14, 15). Different studies showed that unusual DNA methylation and histone deacetylation are associated with tumorigenesis. Consequently, DNA methyltransferases and histone deacetylases were identified as antitumor agents (16). Therefore, this study was designated to distinguish whether decitabine, in addition to its inhibitory effects on dDNA methyltransferases, can reduce HDAC3 and HDAC7 mRNA expression in N-6 (B-Cell precursor leukemia cell line) and HL-60 (Human promyelocytic leukemia cell line) cancer cell lines.

Materials and Methods

Chemicals

Chemicals such as Decitabine, RPMI 1640, Fetal bovine serum (FBS), penicillin and streptomycin, Thiazolyl Blue Tetrazolium Bromide (MTT), Eva green, DNA safe stain, RNA extraction kit, and, cDNA Synthesis

Kits were purchase from SIGMA Otsuka, America Pharmaceutical Inc., (Thermo scientific, MA, USA), (Gibco, Life Technologies, Thermo Fisher, USA), (Bioidea, Iran), (Solis Biodyne, Estonia), (Biofact, Korea), (RNX-plus solution for total RNA isolation- Sinaclon, Iran), (Thermo Fisher scientific, cDNA Inc. USA), respectively.

Cell culture

Human cancer cell lines, including NALM-6, HL-60, and normal cells (B-cell precursor normal cell line for comparison to NALM-6 and human promyelocytic normal cell line for comparison to HL-60), were received from Pasteur Institute Tehran, Iran. After 24 hours rest, cells were cultured in RPMI-1640 media enriched by 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin at 37 °C and 5% CO₂. The MTT assay determined the treatment dose, and NALM-6 and HL-60 cell lines were treated with 0.05 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, and 100 µM Decitabine for 24, 48, and 72 hours, respectively.

Cell viability assay (MTT test)

The cytotoxicity of cell growth was investigated by the kit MTT assay. HL-60 cell was seeded in a 96-well plate (JetBiofill) at a density of 2×10^4 per well. They were treated with 0.05 µM, 0.1 µM, 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, and 100 µM of DAC for 24, 48, and 72 hours after incubation. For the experiment, 10 µl of MTT (Sigma-Aldrich, USA) reagent at 5^{mg/ml} concentrations was added to each well, and the plates were located in the incubator for 4 hours. In the next step, the culture medium was removed, and then 100 µl DMSO 100 was added to each well and placed in the incubator to dissolve the formazan purple crystals. The absorption of wells at 578 nm is read by the ELISA reader (BioTek, USA), and the percentage growth and life of the cells will be calculated. Finally, the obtained mean of 24, 48, and 72 h were considered IC₅₀, and the treatment dose of the drug, and cells were treated with 1 µM dose, at

least 90% of the cells retained their viability (Fig. 1). Assays were performed in triplicates and repeated three times.

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated using an RNA extraction kit instruction protocol (RNX-plus solution for total RNA isolation, Sinaclon,

Iran). Similarly, cDNA was created using the transcriptor first-strand cDNA synthesis kit (Thermo fisher scientific, cDNA Inc. USA). The expression level was calculated using the $2^{-\Delta\Delta C_q}$ method (17). All experiments were repeated at least three times.

The Primers were used in this work shown in Table 1.

Table 1. the sequences of the primers for HDAC3, HDAC7, and GAPDH.

Gene	Primer Sequence	Product Size, bp
HDAC3	F: 5'-CCA AGA CCG TGG CCT ATT T-3' R: 5'-AAT GCA GGA CCA GGC TAT G-3'	111
HDAC7	F: 5'-GGA CAC CAT GCA GAT CAT TC-3' R: 5'-TGC ACG TCC CAG TCT ACA AT-3'	125
GAPDH	F: 5'- GAGCCACATCGCTCAGACAC -3' R: 5'- CATGTAGTTGAGGTC AATGAAGG -3'	150

Finally, all values were normalized to GAPDH expression levels.

Statistical analysis

Statistical analysis was performed using one-way ANOVA. $p \leq 0.05$ was regarded as significant. All statistical analyses were carried out through Graph Pad Prism 7.00 for Windows (Graph Pad Software, San Diego, California, USA). Data were expressed as the mean \pm (SD) of at least three independent experiments.

Results

The inhibitory effect of Decitabine was evaluated on the HI-60 and NALM-6 cells proliferation, followed by manual cell counting after 24, 48, and 72 hours. The cell viability of the treated groups with 1 μ M of Decitabine partially was decreased cell viability in comparison to untreated (control) groups. Exposure to Decitabine with a concentration of 1 μ M, was decreased cell viability from 99% to 90% after 24 hrs, from 99% to 89% after 48 hrs, and from 99% to 89%

after 72 hrs (Fig. 1).

The HDAC3 gene expression in the NALM-6, HI-60 groups, and control groups were analyzed before and after Decitabine treatment. The results show that the expression of the HDAC3 gene was significantly reduced in HI-60 and NALM-6 cell lines after Decitabine treatment (1 μ M). (Fig. 2).

Moreover, HDAC7 gene expression in NALM-6, HI-60, and normal human blood cells was measured before/after Decitabine treatment. The HDAC7 gene expression was significantly reduced in HI-60 and NALM-6 cancer cell lines after treatment with Decitabine (1 μ M) (Fig. 3).

All groups' real-time products were loaded on the Agarose gel under non-saturating conditions on a 3% Agarose gel and stained with ethidium bromide (Fig. 4).

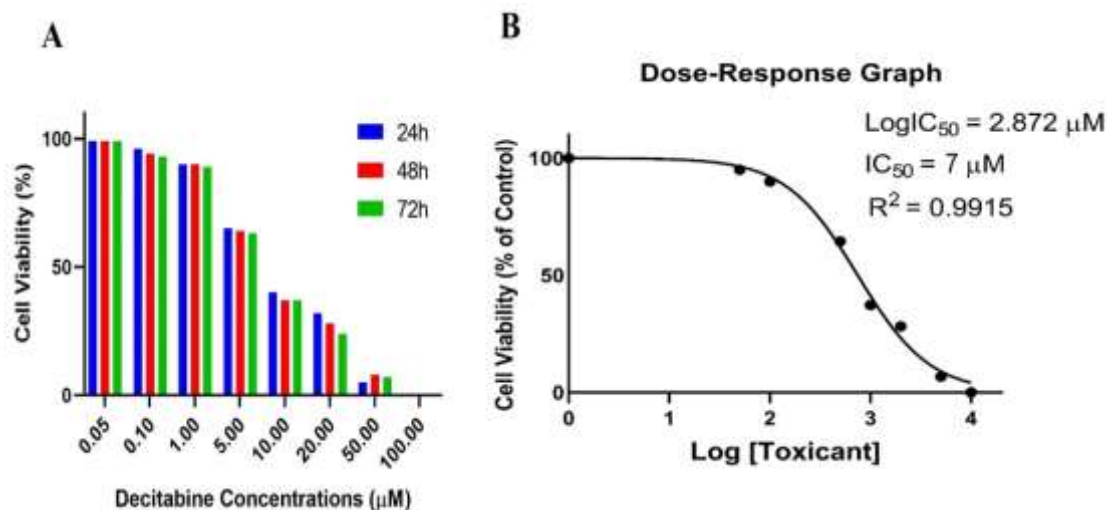


Fig. 1. Cell viability and IC₅₀. A) MTT-assay diagram at different concentrations of decitabine (from 0.05 to 100 µM) for 24 h, 48 h, and 72 h. Cell viability decreased significantly only at concentrations of higher than 1 µM in comparison to control-treated cells.

B) Dose-Response Curve of different concentrations of decitabine. Dose-response curve created by GraphPad prism software follows the familiar symmetrical sigmoidal shape. This model fits a dose-response curve to determine the IC₅₀ (7 µM) of the drug (the

concentration that gives a response halfway between the baseline and maximal). Each cell viability test was performed in triplicate. The treatment group's cell viability was normalized with the Control group's value and was shown as mean values.

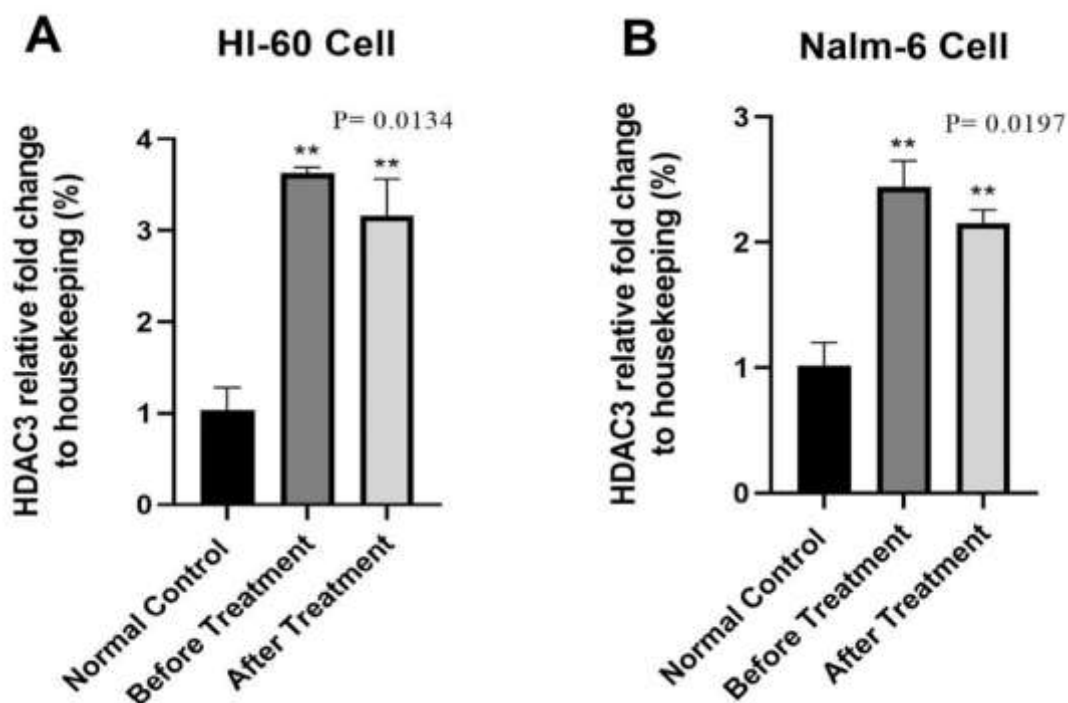


Fig. 2. HDAC3 expression in NALM-6, HI-60, and normal control groups (B-cell precursor normal cell line for comparison with NALM-6 and human promyelocytic normal cell line for comparison with HI-60). A) The expression of HDAC3 gene in the HI-60 cancer cell line and the healthy control group (as calibrator) after normalization to GAPDH. B) the expression of HDAC3 gene in the NALM-6 cancer cell line and the healthy control group (as calibrator) after normalization to GAPDH. After analyzing with one-way ANOVA, the values were considered significant with $p < 0.05$ (**).

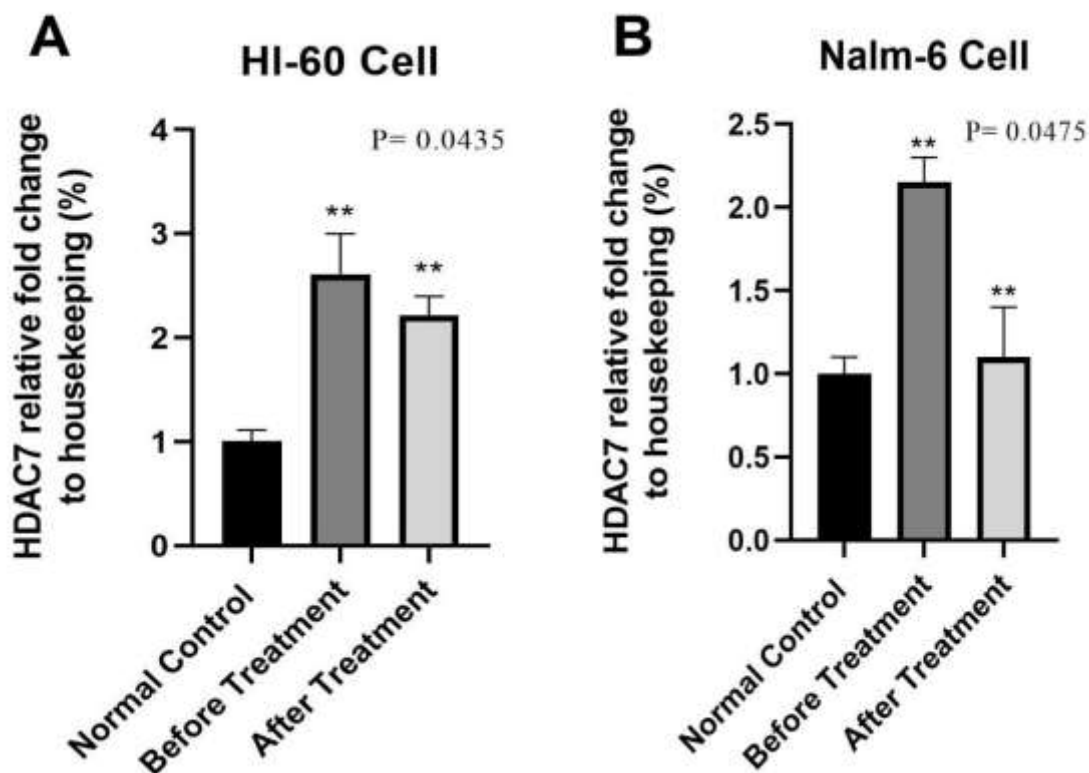


Fig. 3. The chart of HDAC7 gene expression in NALM-6, HI-60, and normal control groups (B-cell precursor Normal cell line for comparison with NALM-6 and human promyelocytic normal cell line for comparison with HI-60). **A)** The expression level of the HDAC7 gene in the HI-60 cancer cell line and the healthy control group (as calibrator) after normalization to GAPDH. **B)** The expression of the HDAC7 gene in the NALM-6 cancer cell line and the healthy control group (as calibrator) after normalization to GAPDH. After analyzing with one-way ANOVA, the values were considered significant with $p < 0.05$ (**).

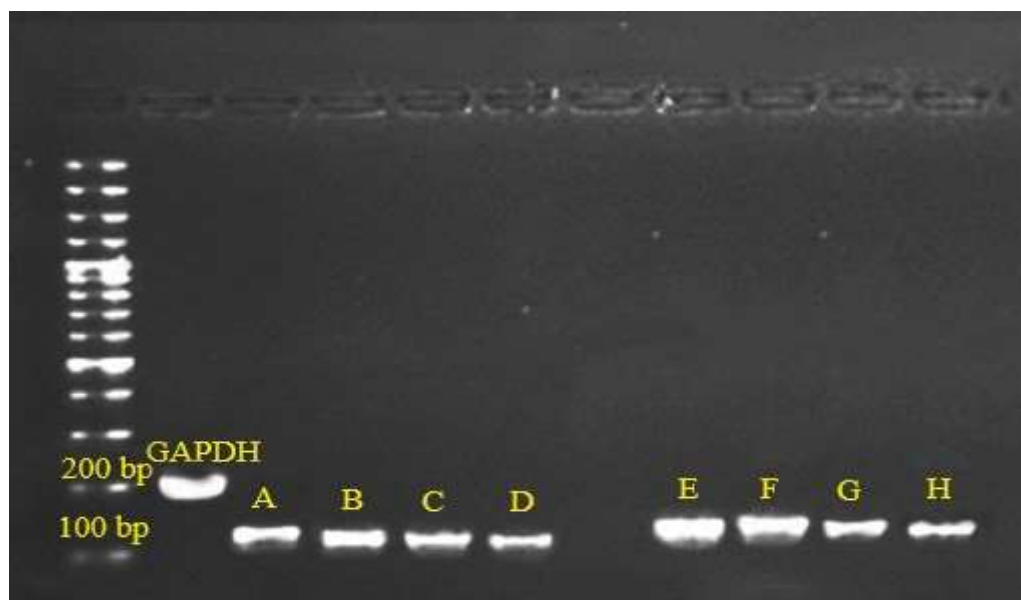


Fig. 4. Real-Time PCR gel patterns (qPCR) of HDAC3 and HDAC7, under non-saturating conditions on a 3% Agarose gel and stained with ethidium bromide. **A)** HDAC3 genes expression in an untreated HL-60 cell line (111 bp), **B)** HDAC3 gene expression in an untreated NALM-6 cell line (111 bp), **C)** HDAC3 gene expression in a treated HL-60 cell line with 1 μ M Decitabine (111 bp), **D)** HDAC3 gene expression in a treated NALM-6 cell line with 1 μ M Decitabine (111 bp), **E)** HDAC7 gene expression in an untreated HL-60 cell line (125 bp), **F)** HDAC7 gene expression in an untreated NALM-6 cell line (125 bp), **G)** HDAC7 genes expression in a treated HL-60 cell line with 1 μ M Decitabine (125 bp), **H)** HDAC7 gene expression in treated NALM-6 cell line with 1 μ M Decitabine (125 bp).

Discussion

Scientific studies show that a low concentration of Decitabine supports the intracellular cycles to remain active (17). In contrast, cellular toxicity associated with higher concentrations of Decitabine is undesirable due to the secondary effect of apoptosis induced by the drug that would interfere with fundamental gene expression changes. A low dose of Decitabine induces hypomethylation, and this effect disappears at high doses (18, 19).

Epigenetic histone alterations play a significant role in chromatin structure. Among enzymes controlling these processes, histone deacetylases (HDACs) eliminate acetyl groups from histone tails, enhancing their interaction with DNA and leading to chromatin condensation (20). Since histone modification, particularly acetylation or deacetylation, is very valuable in the process of chromatin restructuring and genome transcription, so the enzymes involved in histone acetylation modification can be used as therapeutic targets for cancer treatment. Histone deacetylation can alter the transcription of essential genes involved in principal processes, including cell cycle-promoting and regulatory genes, apoptosis, angiogenesis, and cell adhesion by reducing histone acetylation contributing to carcinogenesis. An increased expression of HDACS has been observed in various cancers (21-23).

According to a Karagianni et al. study, enhanced expression of HDAC3 in Acute myeloid leukemia (AML) is correlated with poor prognosis and response to treatment (21), and in the study by Moreno DA et al., enhanced expression of HDAC3 has also been in childhood acute lymphoblastic leukemia (ALL) (21). Overexpression of HDAC 3 in several cancer types has been correlated with poor prognosis and response to treatment, including prostate, breast, ovarian and colorectal cancers, and T-cell acute lymphoid leukemia (T-ALL) (22, 24, 25). Several studies have shown that overexpression of HDAC7 and its interaction with MEF2 family genes in ALL mostly inhibits transcription in lymphoid cells, causing

normal differentiation failure in the lymphoid lineage. Overexpression of HDAC7 is correlated with a weak prognosis in chronic lymphoid leukemia (CLL). It binds to the c-Myc transcription factor gene, causing its overexpression and enhanced proliferation of cancer cells in CLL (26, 27).

Moreno et al. Found that in ALL (Acute lymphoblastic leukemia), significantly increased HDAC2, HDAC3, HDAC6, HDAC7 and HDAC8 gene expression was observed. Similarly, in our study, the expression of HDAC3 and HDAC7 genes in NALM-6 (human B cell precursor leukemia) and HI-60 (human promyelocytic leukemia) cancer cell lines was significantly increased compared to healthy cells. It has also been shown that HDAC3, HDAC7, and HDAC9 gene expression are increased in childhood ALL. Histone deacetylase inhibitor medicines alter some genes' expression and eventually induce apoptosis in cancer cells by adjusting the histone acetylation and modifying and non-histone proteins (26, 28-31).

So far, Decitabine has been considered more as a hypomethylating agent (32-37) and less as a histone deacetylase inhibitor. This study investigates the histone deacetylase inhibitory status of Decitabine. In this research, Decitabine significantly reduced HDAC3 and HDAC7 genes' expression compared to the untreated group and showed a decreasing effect on HDACs. Based on our results, although Decitabine is a weaker histone deacetylase inhibitor than the specific inhibitor of histone deacetylase such as TSA (Trichostatin A) (9), Decitabine Has both features of potent hypomethylating and a reducer of histone deacetylase expression so that it can be unique in this respect. However, the last feature needs to be confirmed by proteomics and clinical experiments.

It is also suggested that the inhibitory effect of Decitabine evaluated on other histone deacetylases, such as HDAC2, HDAC6, HDAC8, and HDAC9, which increased in cancers. Decitabine is a powerful

hypomethylating factor and Reducing HDAC3 and HDAC7 mRNA expression. Multiple therapies cause various side effects in patients, and the use of multiple anticancer drugs is restricted due to toxicity and the increase of drug resistance (38). So, new drugs are needed to ameliorate the clinical consequence and decrease chemotherapy endurance (39). Therefore, Decitabine can use as a candidate for hypomethylating and HDAC3 and HDAC7 reducing agents.

Moreover, increasing information about the negative role of histone deacetylase overexpression in various cancers could help to produce new drugs that can simultaneously target methylation and acetylation in cancer cells.

In summary, the current study results propose the possible involvement of HDAC3 and HDAC7 in leukemias. Additional investigations are suggested to elucidate the

potential role of HDAC3 and HDAC7 in carcinogenesis. These data showed that the effects of Decitabine are not limited to direct hypomethylation of DNMTs and may indirectly affect other epigenetic factors, such as HDACs activity, through converging pathways.

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