Harmine, a Novel DNA Methyltransferase 1 Inhibitor in the Leukemia Cell Line

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Abstract DNA methylation followed by tumor suppressor gene repression plays a critical role in the leukemia development. So, DNA methyl transferase inhibitors have great importance in treatment of these malignancies. Harmine, A beta carboline alkaloid derivative of Peganum harmala, had shown anti-proliferative effects on leukemic cell line. This study aimed to evaluate the effect of Harmine on DNMT1 (DNA methyl transferase 1) expression in a leukemic cell line. Cell proliferation and cell cycle analysis were studied in NB4 cell line after treatment with Harmine for 72 h. DNMT1 expression in treated cells was analyzed by real time PCR. Tumor suppressor gene hypomethylation and reactivation was evaluated via MSP analysis and also real time PCR. Harmine reduced cell proliferation in NB4 cell line in a time and dose-dependent manner. 102 µg/ml of Harmine was increased amount of cells in G1 Phase of cell cycle ($p < 0.05$). Anti-proliferative doses of Harmine, has suppressed DNMT1 gene in NB4 cell line. Down-regulated DNMT1 induced p15 tumor

suppressor promoter hypomethylation and reactivation. Our data indicate that Harmine can be considered as a potential treatment for AML (Acute Myeloid Leukemia), and future studies are required to test the clinical efficacy of Harmine—whether used as a single agent or as an adjuvant—for AML treatment.

Keywords Harmine · Leukemia · Dnmt1 · P15

Introduction

In many tumors, including solid and hematologic malignancies, aberrant hypermethylation of CpG-rich regions upstream of tumor suppressor genes (TSG) lead to the recruitment of repressive complexes and transcription inactivation [1, 2]. DNA methylation is performed by a family of DNMTs (DNA methyl transferases) of which three main members have been identified in eukaryotic cells: DNMT1, DNMT3A, and DNMT3B. DNMT1 is considered a maintenance methyltransferase, whereas DNMT3A and DNMT3B catalyze de novo methylation of DNA. DNMT1 which catalyzes the transfer of methyl groups to DNA provides a crucial mediator of DNA methylation. [3].

DNA methylation is a reversible process, thus made it an attractive target for cancer therapeutics. Inhibition of DNA methylation is an effective treatment for leukemia and myelodysplastic syndrome. There are two main (FDA)-approved DNA hypo-methylating agents, 5-azacitidine (5-aza) and decitabine (5-aza-deoxycytidine), which have proven effective in treating human tumors [3].

These compounds are incorporated into RNA or DNA and inhibit the DNA methyltransferases (DNMTs), thereby allowing re-expression of tumor suppressor genes (e.g.

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P15, P21) and final restoration of normal cell cycle, proliferation and cell differentiation [4, 5].

However, Aza-nucleosides drugs are relatively non-specific and have some limitations on use. These drugs are active only in S phase of cell cycle and they have limited efficacy for hypo proliferate cancer cells. Also, there are some unwanted side effects due to their relative non specificity, [6] so with respect to the restrictions on the use of these synthetic DNMT inhibitors, the discovery and development of a new generation of inhibitors would seem to be necessary. [7] Therefore, the natural inhibitors of DNA methylation, such as: Curcamin [8, 9], Genistein [10], Acid Galic [11], Catechin [12] and Epicatechin [13] have been identified that are capable of inducing hypomethylation and de-methylation activity [14].

Peganum harmala, that had been used in traditional medicine as an anti-inflammatory and hypothermia agent [15] contain about 2–6% pharmacological active alkaloids [16], which are mostly b-carbolines such as Harman, Harmine, Harmaline and Harmalol [17]. Harmine, a β -carboline alkaloids originally isolated from the seeds of Peganum harmala possess a broad range of pharmacological activities, such as behavioral effects [18]. Recent studies have demonstrated, significant anti-tumor potential of Harmine both in vitro and in vivo [19], e.g. significant tumor inhibition in sarcoma180 or Hep-A tumor [20], broad cytotoxicity spectrum against human lung carcinoma cell lines [21] and leukemic cell line [22].

In this study, we examine the impact of Harmine on DNMT1 expression, hypo methylation of p15 tumor suppressor gene promoter and reactivation in leukemic cell lines.

Materials and Methods

Cell Culture Conditions and Reagents

NB4 cells, the human promyelocytic cell line, obtained from Institute of Biochemistry and Biophysics at Tehran University. 1×10^6 cells/ml were cultured in RPMI (Sigma, California, USA) supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 μ g/ml streptomycin (Sigma, Saint Louis, USA) at 37 °C in a humidified atmosphere, 5% CO₂ in the air. Harmine (Sigma, Saint Louis, USA HPLC grade) and 5-azacytidine (Sigma, Saint Louis, USA) was added to the cells in multi well plates and incubated for 5 days. Harmine and 5-azacytidine (control positive) dissolved in ethanol.

In Vitro Cell Proliferation

NB-4 cells (3×10^5 cells), were treated with Harmine at (102.4, 51.2, 25.6, 12.8, 6.4 μ g/ml) in a 6-well plate for

3 days. Cell growth was assessed by means of manual cell count on a hemocytometer using trypan blue (Sigma, Saint Louis, USA) at 24, 48, and 72 h. Liquid 0.4% trypan blue was purchased from Sigma ready to use.

MTT Assay

Effect of different concentration of Harmine on cell proliferation was assayed by MTT colorimetric assay. The cell suspension was dispensed into a 96-well tissue culture plate. Harmine at various dilutions (102.4, 51.2, 25.6, 12.8, 6.4, 3.2 and 1.6 μ g/ml) were added into micro plate wells. After incubation at 37 °C for 72 h, 5 mg/ml MTT (ATO-CEL, Budapest, Hungary) in PBS (Sigma, Saint Louis, USA) was added into each well. The cells were incubated for 4–6 h at 37 °C in the dark. At the end of the incubation period, DMSO (Merck, Germany) was added to dissolve the blue formazan product, and optical density (OD) was measured at 570 nm using a microplate Elisa reader.

Cell Cycle Distribution Analysis by Flow Cytometry

Apoptosis ratings and Cell cycle distribution were evaluated after 72 h of culture by flow cytometry (Partec, Germany). Prior to staining, 1×10^6 cells/ml were washed and suspended in 200 μ l of PBS. Cells were treated with 50 μ l of RNase (Sigma, Saint Louis, USA) (1 mg/ml) and 100 μ l Propidium iodide (PI, 400 μ g/ml) (Sigma-Aldrich, Spain) for 30 min at 37 °C in the dark. The fluorescence of stained cells (Intra cellular staining) was analyzed in a flow cytometry and relative gated cells in each cell cycle were determined. Data acquisition and analysis were performed using the flow Macs 2.0 software.

Samples and Extraction of DNA and Total RNA

DNA and RNA were extracted from treated and untreated NB4 cells after 72 h of culture. They were immediately frozen at -70 °C until use. DNA was isolated by a QIAamp DNA Mini-kit (Qiagen, Valencia, CA, USA), and RNA was extracted using Trizol reagent (Roche, Mannheim, Germany).

Expression Analysis and Quantitative Real-time PCR

Reverse transcription was performed on 1 μ g total RNA, with AccuPower CycleScript RT PreMix reverse transcription Kit (Bioneer, USA). The reaction was carried out in 12 cycles of 22 °C for 30 s, 45 °C for 4 min; 55 °C for 30 s, and 1 cycle of 95 °C for 5 min. Products were analyzed in 1.5% agarose gel under UV light. Quantitative RT-PCR was performed with the LightCycler technology,

using 3 μ L cDNA in 25 μ L reaction volume with 0.4 μ M of each primer and 12.5 μ L of 2 $\times$ Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals, Germany).

The forward primer for Dnmt1 was 5'-CCTCCAAAAACCCAGCCAAC -3', reverse primer was 5'-TCCAGGACCCTGGGGATTTC -3' and the PCR product was 101 bp. The sequences of p15 INK4B primers were: forward 5'-GGG AGGGTA ATG AAG TTG AGT TTA-3', and reverse 5'- ACC CTAAAA CCC CAA CTA CCT AAA T -3'.

Thermal cycling was initiated at 95 °C for 5 min followed by 40 cycles of PCR (95 °C, 30 s; 57 °C, 30 s; 72 °C, 20 s). GAPDH was used as an endogenous control. The fold change ratio was calculated via $2^{-\Delta\Delta C_t}$ method.

Bisulfite Modification and Methylation-Specific Polymerase Chain Reaction

DNA was subjected to bisulfite modification using an EpiTect Bisulfite kit (Qiagen, Valencia, CA, and USA). Modified DNA was purified, re-suspended in distilled water, and stored at -20 °C until use. Human genomic DNA was used to determine the bisulfite conversion efficiency.

Methylation-Specific Polymerase Chain Reaction (MSP)

The modified DNA template was used to amplify the p15INK4b promoters with methylated and unmethylated-specific primer pairs, of which the sequences have been chosen from promoter regions containing frequent cytosines, and CpG pairs near the 3 end of the primers to provide discrimination in the polymerase chain reaction (PCR) between methylated and unmethylated DNA [23, 24]. Primer sequences for the p15 gene: unmethylated reaction was as follows: 5'-TGTGATGTGTTTGTATTTTGTGG TT-3' (sense) and 5'-CCATACAATAACCAAAACAA CCAA-3' (antisense); and for methylated reaction was as follows: 5'-GCGTTCGTATTTTGCGGTT-3' (sense) and 5'-CGTACAATAACCGAACGACCGA-3' (antisense).

The PCR was performed using 3 μ L DNA (approximately 100 ng) in 25 μ L reaction volume with 0.4 μ M of each primer, 10 $\times$ pcr buffer with Mg⁺ (Roche, Mannheim, Germany) and 2.5 mM dNTP (Roche, Mannheim, Germany). PCR amplification of the modified DNA samples consisted of 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 30 s; and 1 cycle of 72 °C for 10 min. Each modified DNA sample was amplified by unmethylated and methylated primers with 150-bp PCR product length band. Bisulfite-converted methylated and unmethylated EpiTect PCR DNA (Qiagen, Valencia, CA, and USA) was used as controls. The

products were separated by electrophoresis on 2% agarose gel. The results were always confirmed by repeated MSP analyses.

Results

Effect of Harmine on Cell Proliferation

Marked changes in the proliferate properties of NB4 cells were observed during incubation with Harmine. The optimal doses for anti proliferate effect were chosen (102.4, 51.2, 25.6, 12.8, 6.4, 3.2, 1.6 μ g/ml). MTT assay showed at 1.6, 3.2, 6.4 and 12.8 μ g/mL of Harmine, cell viability was 88, 78, 70, 58% respectively. But at higher doses of Harmine including 25.6, 51.2, 102.4 μ g/ml, viability intensively decreased (50, 28, 18%) ($p < 0.05$). The assay showed Harmine at 25.6 μ g/ml, led to 50% decreasing in viability as compared with control ($p < 0.05$) (Fig. 1).

Cell Cycle Distribution Analysis by Flow Cytometry

Cell cycle analysis was performed by PI staining of NB4 cells treated with Harmine at 102.4, 51.2, 25.6 μ g/ml and 5-azacytidine (2 μ Molar) after 3 days of culture. The analyses of cell cycle showed arresting of cells in G0/G1 phase at 102.4 and 51.2 μ g/ml of Harmine ($p \leq 0.05$), but 5-azacytidine (positive control) led to arrests of cells in S phase (Fig. 2).

DNMT1 Expression in NB4 Cells Under Harmine Treatment

Expression of DNMT1 in NB4 cells under treatment with Harmine after 3 days was evaluated by Real time-PCR.

Fig. 1 Proliferation analysis of in NB4 cells under treatment with Harmine by MTT assay (n = 3), ($p < 0.05$)

Fig. 2 Cell cycle analysis of NB4 cells under Harmine treatment, RN1 = G0/G1 RN2 = G2/M RN3 = S. **a** Untreated NB4 cells **b** 5-Azacytidine (2 µM) **c** Harmine (102.4 µg/ml) **d** Harmine (51.2 µg/ml)

The expression of DNMT1 was decreased (83, 69, 59, 86%), at 102.4, 51.2 and 25.6 µg/ml of Harmine (≤ 0.05) and 2 µMolar of 5-azacytidine (control positive) respectively (Fig. 3).

Methylation Status of the p15INK4b in NB4 Cells Under Harmine Treatment

We examined the methylation status of p15INK4b gene promoter in NB4 cells under treatment by 102.4 and 51.2 µg/ml doses of Harmine after 3 days. The effect of these doses of Harmine on NB4 cell line showed hypomethylation in p15 gene promoter (Figs. 4, 5).

mRNA Expression of p15INK4b in NB4 Cells Under Harmine Treatment

Expression of p15INK4b in NB4 cell under treatment with Harmine (102.4, 51.2 µg/ml) and 5-azacytidine (2 µM)

Fig. 3 DNMT1 expression ratio under 72 h Harmine (102.4, 51.2 and 25.6 µg/ml) and 5-azacytidine (2 µMolar) treatment (n = 3), ($p < 0.05$)

after 72 h incubation was evaluated by Real time PCR. The result showed that the fold change ratio of P15 mRNA expression in the NB4 Cell at doses (102.4, 51.2 µg/ml) was 17-fold and 13-fold respectively (Fig. 5).

Fig. 4 Methylation-specific PCR of bisulfite-modified DNA using primer pairs specific for methylated and unmethylated p15INK4b promoter sequences **a** Untreated NB4 cells. Lane 1 is DNA ladder; Lane 2 is untreated NB4 cells with the methylated primer; Lane 3 is untreated NB4 cells with the unmethylated primer. **b** Treated NB4 cells with Harmine (102.4 and 51.2 µg/ml). Lane 1 is Harmine (102.4 µg/ml) treated NB4 cells with the methylated primer; Lane 2 is Harmine (102.4 µg/ml) treated NB4 cells with the unmethylated primer; Lane 3 is Harmine (51.2 µg/ml) treated NB4 cells with the unmethylated primer; Lane 4 is Harmine (51.2 µg/ml) treated NB4 cells with the methylated primer; Lane 5 is Control DNA with the unmethylated primer; Lane 6 is Control DNA with the methylated primer; Lane 7 is DNA ladder

Discussion

Leukemia, as any other cancer, displays aberrant methylation in the tumor suppressor genes; these TSGs were associated with prognosis and prediction of treatment

Fig. 5 p15 expression ratio under 72 h Harmine (102.4 and 51.2 µg/ml) and 5-azacytidine (2 µMolar) treatment. (n = 3), (p < 0.05)

response [25]. As abnormal methylation of the promoters is potentially reversible, pharmacological inhibitors of DNA methylation provide a rational approach to restore the cellular functions that are abnormally silenced by hypermethylation [26]. In this study Harmine, as an antiproliferate agent [19–22], was evaluated for DNA methylator inhibition. Harmine could inhibit cell proliferation in NB4 cell line at all doses tested in a time and dose-dependent manner. Similar results were obtained with previous reports in different types of tumor, such as sarcoma, lung cancer [27], and myeloid leukemia [22].

We demonstrated that, in response to Harmine treatment (51.2 µg/ml), NB4 cells became arrested at the G1 phase of the cell cycle. Hamsa et al. [28] also concluded that Harmine at very low concentrations leads to arrest cells in the G0G1 phase.

We also found that Harmine (25.6 µg/ml) decreases DNMT1 mRNA expression in NB4 cell line. This down-regulation of DNMT1 expression was concomitant with p15INK4B tumor suppressor gene reactivation, hypomethylation of the p15INK4B promoter. All of these events may occur together, and subsequently contribute to anti-leukemic effects of Harmine in AML.

In contrast to hypomethylators like 5-azacytosides (e.g. Decitabine), which are only useful during S phase and are used only for the Hyper-proliferative hematologic malignancies [29, 30]; Harmine could effect in G1 phase of cell cycle.

There are a number of additional limitations for the use of azanucleosides. In particular, these drugs can cause myelo-suppression [29] and are unstable; furthermore azanucleosides can be degraded via hydrolytic cleavage and de-amination by cytidine de-aminase [31].

In contrast, novel anti tumor agents from natural resources such as curcamine [14], Epigallocatechin-3-gallate [32] and Mahanine [33] represent alternative hypomethylating agents which may be devoid of several of these limitations. Therefore, might serve as a complementary therapy, in the setting of azanucleoside-resistance, during treatment of leukemia.

Jianhua Yu et al. [14] observed that Curcamine (10 and 20 μ M) decreased Dnmt1 gene expression in MV4-11 cell line. Soumik Agarwal et al. in 2013 showed that 10 μ M Mahanine reduced DNMT1 expression in prostate cancer cells [33]. In our Study Harmine (25.6 μ g/ml) induced DNMT1 gene suppression in NB4 cells in a time and dose dependent manner.

Down-regulated DNMT1 induced p15 tumor suppressor gene promoter hypomethylation and reactivation (Harmine, 51.2 μ g/ml). Jianhua Yu et al. [14] observed that Curcumin (10–20 μ M) dramatically increased p15INK4B mRNA expression in two leukemic cell lines. Mayada Achour et al. [32] in 2012 showed that Epigallocatechin-3-gallate (30 μ g/ml) caused Hypomethylation in p16, TSGL, and P73 promoter in Jurkat cells and increased expression of these genes.

Conclusion

Our findings indicate that Harmine down-regulates DNMT1 expression, which results in DNA hypomethylation and reactivation of one TSG in AML, and also correlates to its role as an inhibitor of tumor growth, so Harmine can be considered as a potential treatment for AML, and future studies is required to test the clinical efficacy of Harmine—whether used as a single agent or as an adjuvant—for AML treatment.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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