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ORIGINAL ARTICLE

Expression analysis of genes involved in epigenetic regulation and apoptosis in human malignant haematopoietic cell lines treated with 5-azacytidine

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Summary

Purpose: *The aim of this study was to investigate the modulation of the expression status of 10 different genes involved in epigenetic regulation and apoptosis by the DNA methyltransferase (DNMT) inhibitor 5-azacytidine (5-Aza), as markers of response to treatment, in two different human malignant haematopoietic cell lines.*

Methods: *In our analysis we used the SybrGreen technology and gene-specific primers for the qRT-PCR analysis of 10 genes, in cDNA of PC-MDS and K562 cell lines, treated by 1 micromole of 5-Aza for 24h.*

Results: *DNMT1 and DNMT3A showed statistically significant decrease of expression in 5-Aza-treated PC-MDS cells, whereas DNMT3B showed significantly decreased expression in 5-Aza-treated K562 cells. The members of the Bcl-2 family of apoptosis-regulating genes Bcl-2 and Bax showed statistically significant differences in expression, in comparison with non-treated PC-MDS cells. Our most interesting result was the significant upregulation (re-expression) of p15, in 5-Aza-treated PC-MDS cells.*

Conclusion: *The re-expression of p15 in PC-MDS cell line evaluated by qRT-PCR makes this novel cell line a suitable model for the studies of pharmacologic demethylation as a plausible mechanism resulting in hematologic response in myelodysplastic syndrome (MDS).*

Key words: 5-azacytidine, demethylation, myelodysplastic syndrome

Introduction

The incidence of MDS in the USA is 3.4 per 100,000 population [1]. With 40,000-76,000 new cases per year in the USA, MDS is the commonest of the hematological disorders and carries a significant burden of morbidity and premature death [2]. Epigenetic changes have been increasingly recognized as a molecular basis underlying the development of human leukemia and MDS. Abnormal methylation appears to accumulate over time at various sites in the genome and promotes tumorigenesis by increasing genomic instability or by silencing tumor suppressor genes [3]. Silencing of tumor suppressor genes is closely associated with hypermethylation; methylated tumor suppressor genes can be reactivated by DNMT inhibitors. These observations have led to a revival of interest in DNA methylation inhibitors as antineoplastic agents in clinical trials. The prototypic DNMT inhibitors 5-azacytidine (5-Aza) and 5-aza-2-deoxycytidine (DAC) have recently been approved by the US Food and Drug Administration as antitumor agents for the treatment of MDS [4,5]. Translational research represents the effective transfer of molecular research of anticancer drugs into their effective clinical application, emphasizing the need for suitable *in vitro* models for MDS [6-10].

5-Aza is a cytidine analog capable of altering the expression of certain genes, and to reactivate tumor suppressor and DNA repair genes through hypomethylation of cytosines. In recent years, a better understanding of the biology of MDS has emerged with the recognition that aberrant epigenetic silencing of key genes may be contributing to the pathogenesis [2,11]. One mode of gene silencing is methylation, a reversible process that may be targeted by selective agents such as 5-Aza and decitabine. In addition to cytotoxic effects, 5-Aza in-

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duces differentiation of malignant cells *in vitro*. The phosphorylated and reduced metabolite of 5-Aza inhibits DNA methyltransferase, the mammalian cells enzyme responsible for methylating newly synthesized DNA, resulting in the synthesis of hypomethylated DNA and changes in gene transcription and expression [2,12].

The prototypic nucleoside analogue DNMT inhibitor 5-Aza is incorporated into DNA and RNA, and forms a covalent complex with the DNMT enzyme resulting in the trapping and degradation of the enzyme and progressive loss of DNMT activity in cells [13]. In mammalian cells, the enzymes critical for DNA methylation reactions are known as DNA cytosine-5-methyltransferases (DNMTs), the most studied among them being DNMT1. DNMT1 prefers semimethylated DNA as a substrate and therefore will methylate the newly replicated DNA only when the template nucleotides are methylated. DNMT1 is constitutively expressed and is required to maintain global methylation after DNA replication has taken place. Recently, other enzymes with the ability to methylate DNA have been identified, including DNMT3A and DNMT3B, which appear to be involved in *de novo* methylation, that is, methylation which involves the addition of methyl groups to sites not previously methylated [14,15]. Global hypomethylation associated with high transcriptional levels of these enzymes has been observed in MDS and ovarian cancer [16,17]. DNMT inhibitor 5-aza-2-deoxycytidine affects the transcription of DNMT1 and DNMT3A in a cell type-depending manner [18]. Therefore, we were interested to test the 5-Aza effects on transcriptional levels of mRNA of 3 DNMT isoenzymes in PC-MDS and K562 cell lines.

The DNA demethylating agents are able to reactivate silenced tumor suppressor genes such as the cyclin-dependent kinase inhibitor p15/INK4b *in vivo*. However, it is most likely that also the regulation of other genes is associated with its activity. One possible mode of action of azanucleosides is the modification of a group of genes encoding the proteins involved in epigenetic regulation and apoptosis, such as members of the Bcl-2 family of apoptosis-regulating genes [19].

Azanucleosides are established molecular tools for the induction of DNA demethylation in cellular model systems. One potential problem with both agents is that resistance can develop during treatment [4]. However, it is also known that high doses of these drugs can induce pronounced toxicities in patients. In this context, novel MDS cell lines, such as PC-MDS may be a useful *in vitro* model for drug response testing.

The aim of our study was to investigate the modulation of the expression status of 10 different genes involved in epigenetic regulation, DNA repair and apoptosis by the DNMT inhibitor 5-Aza, as markers of response to treatment in MDS cell line.

Methods

Gene expression experiments were performed in cDNA of PC-MDS and K562 (ATCC CCL-243 chronic myelogenous leukemia) cell lines, treated by 1 micromole of 5-Aza for 24h. The PC-MDS cell line is a novel cell line derived from the bone marrow of a patient with therapy-related myelodysplastic syndrome (t-MDS) who had no overt post-MDS leukemia. Classic cytology analyses, immunophenotyping, cytogenetic and molecular genetic procedures were used for characterization of the cell line, as previously published [20,21].

Cells of both human malignant haematopoietic cell lines, PC-MDS and K562 were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 2 mM glutamine, 10% FCS (PAA Lab GmbH Pasching, Austria) and antibiotics (penicillin and streptomycin, Galenica, Belgrade, Serbia), at 37°C in fully humified atmosphere with 5% CO_2. Azacytidine (5-Aza, Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile water. PC-MDS and K562 cells were incubated with 5-Aza final concentrations of 0.1, 0.5, 1, and 2 μM (100 nmol/l; 500 nmol/l; 1000 nmol/l and 2000 nmol/l) for 8, 12, 24, and 48 h.

Cytotoxicity of 5-Aza in PC-MDS and K562 cell lines was analyzed by a colorimetric assay using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cells were plated in 96-well plates, allowed to grow overnight and treated with 0, 0.1, 0.5, 1 and 2 μM 5-Aza for 8, 12, 24 and 48h. Three hours before the end of each experiment, 10 μl of 5 mg/ml MTT solution was added into each well of 96-well plates. The supernatant was discarded and 100 μl of 0.04 mol/l HCl in isopropanole was added to dissolve the blue insoluble MTT formazan. The plate was mixed gently until the blue sedimentation was completely dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340, Finland) at a test wavelength of 540 nm and a reference wavelength of 690 nm. Wells containing the medium and MTT but no cells were used to blank the plate reader. Data were expressed in optical density (OD) units.

RNA extraction from both cell lines was performed by the RNeasy Mini Kit (QIAGEN, USA). The quality of RNA was analyzed by the Bioanalyser 2100 (Agilent, USA). The synthesis of cDNA was performed using the 1 μg of K562 and PC-MDS RNA as a tem-

plate, using the cDNA synthesis kit (Superscript III, Invitrogen, USA). For the reverse transcription, we used the combination of the oligo dT and random hexamers, in order to achieve the optimal cDNA synthesis.

Primers were designed using Primer Express 3.0 Software (Applied Biosystems, USA) and produced by Sigma (the sequence of primers for all 10 genes is shown in Table 1). For qRT-PCR, the primers were used in concentration of 1.2 μM, each in a total qRT-PCR volume of 20 μl. qRT-PCR analysis was performed using the instrument StepOne Plus (Applied Biosystems, USA) for quantification with Sybr Green and the standard software PCR product fluorescence detection and analysis (Applied Biosystems StepOneTM Real-Time PCR software v. 2.0). Relative mRNA expression levels of all the examined genes and gene transcripts were determined from the threshold cycle values and were normalized using the human ACTB gene as a reference gene (Applied Biosystems, USA) to yield the relative abundance.

Statistical analysis

All data were expressed as the mean values of triplicate measurements. Results were expressed as mean ± standard deviation of the mean (SD). Differences among means for 5-Aza treated and non-treated cells were tested for statistical significance by Student's two-tailed t-test. Statistical significance was set at $p<0.05$.

Results

The cytotoxicity of 5-Aza in PC-MDS and K562 cell lines showed dose and time-dependent effects (supplementary Figures 1A and 2A). Cells were incubated with 0.1, 0.5, 1 and 2 μM of 5-Aza for 24 h. 5-Aza concentrations of ≥ 0.5 μM induced increase in the percentage of cellular toxicity at this time point in comparison with non-treated cells. As shown in Figures 1B and 2B, prolonged incubation from 24 to 48 h did not increase the cytotoxicity of K562 and PC-MDS cell lines. We have selected 1 μM of 5-Aza and 24h treatment for gene expression analyses, showing that this selected regime achieves suppression of cell growth, but not significant cellular toxicity. The cytotoxicity of 1 μM of 5-Aza for 24h was significantly more pronounced ($p <0.05$) in K562 in comparison with PC-MDS cell line (Figure 1A).

To determine the effect of 5-Aza on gene expression in myeloid leukemia cell lines, PC-MDS and K562

Table 1. List of primers for qRT-PCR expression analysis

	Name of primer	*Sequence of primer*	*Name of gene*
1	P15INK4b_F	CGGGGACTAGTGGAGAAGGT	CDKN2B, cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
	P15INK4b_R	GGTGAGAGTGGCAGGGTCT	
2	DNMT1_F	GAGGAGCCGGACAGAGAAG	DNMT1, DNA (cytosine-5-)-methyltransferase 1
	DNMT1_R	TTCATCCTCGTCTTTTTCATCA	
3	DNMT3A_F	TCCAAAGGTTTACCCACCTG	DNMT3A, DNA (cytosine-5-)-methyltransferase 3 alpha
	DNMT3A_R	GTCCCCGACGTACATGATCT	
4	DNMT3B_F	TGTAATCCAGTGATGATTGATGC	DNMT3B, DNA (cytosine-5-)-methyltransferase 3 beta
	DNMT3B_R	GGCTATCCTATTGTATTCCAAGCA	
5	Bcl-2_F	GGTGACCAGTTCAACGGAGA	Bcl-2, B-cell CLL/lymphoma 2
	Bcl-2_R	CCAGGAATCAATCACCCAAT	
6	Bax_F	ACCAAGAAGCTGAGCGAGTG	Bax, BCL2-associated X protein
	Bax_R	CCTCCCAGAAAAATGCCATA	
7	Bak_F	GTAGCCCAGGACACAGAGGA	BAK1 BCL2-antagonist/killer 1
	Bak_R	GTCAGGCCATGCTGGTAGAC	
8	hOGG1_F	GCAGCAGCTACGAGAGTCCT	hOGG1, 8-oxoguanine DNA glycosylase
	hOGG1_R	AGGGTGCCAGCTGTAGTCAC	
9	Baxi_F	CGGACTCTGGAACCATGAAC	Bax-inhibitor
	Baxi_R	GCAAATCCAGCAAGAAGTCC	
10	SOD_F	CGTCACCGAGGAGAAGTACC	SOD, superoxide dismutase
	SOD_R	CTGATTTGGACAAGCAGCAA	

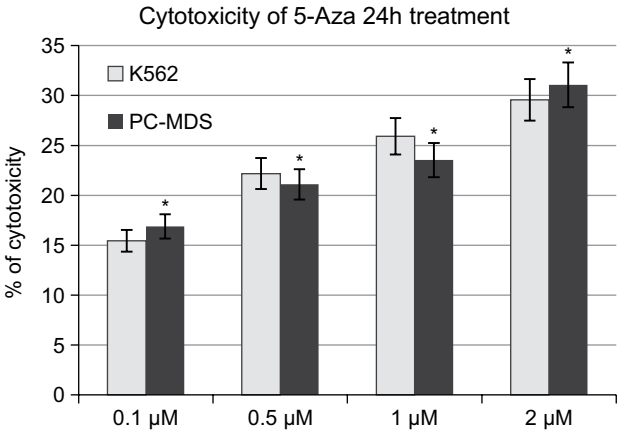

Figure 1A. Cytotoxicity of 5-Aza 24h treatment. Dose-dependent effects of 5-Aza in 24h treated PC-MDS and K562 cell lines. *p<0.05.

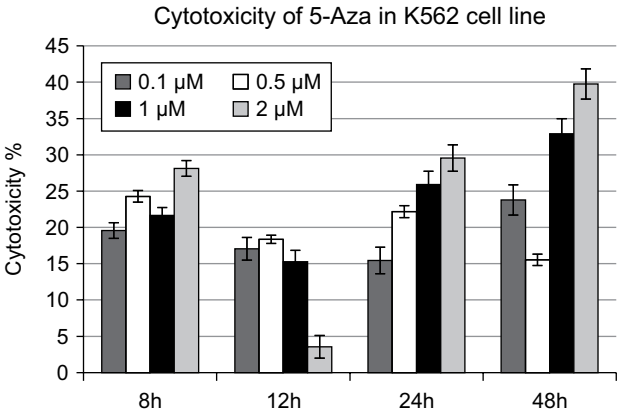

Figure 1B. Cytotoxicity of 5-Aza in K562 cell line. Time and dose-dependent effects of 5-Aza in K562 cell line.

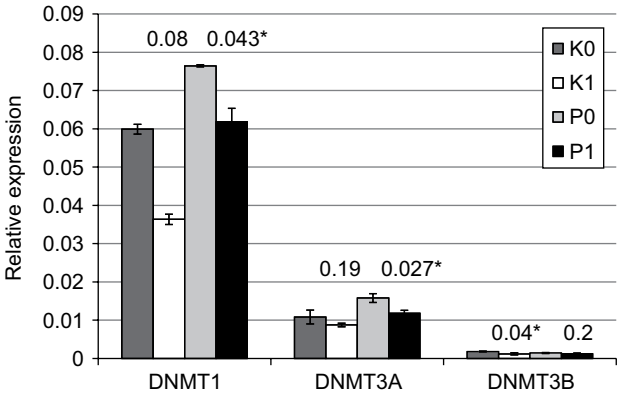

Figure 3. Relative expression levels of DNMT1, DNMT3A and DNMT3B mRNA in K562 and PC-MDS cell line, treated by 1 μM 5-Aza for 24 h (K0=K562 control; K1=K562 1 μM 5-Aza for 24 h; P0=PC-MDS control; P1=PC-MDS 1 μM 5-Aza for 24 h). *p<0.05.

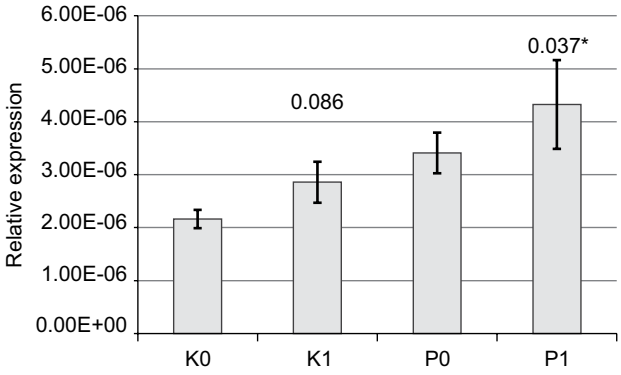

Figure 2A. Relative expression levels of p15 mRNA in K562 and PC-MDS cell line, treated by 1 μM 5-Aza for 24 h (K0=K562 control; K1=K562 1 μM 5-Aza for 24 h; P0=PC-MDS control; P1=PC-MDS 1 μM 5-Aza for 24 h). *p<0.05.

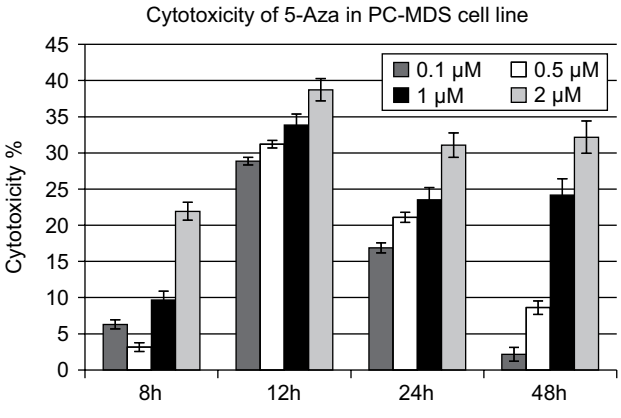

Figure 2B. Time and dose-dependent effects of 5-Aza in PC-MDS cell line.

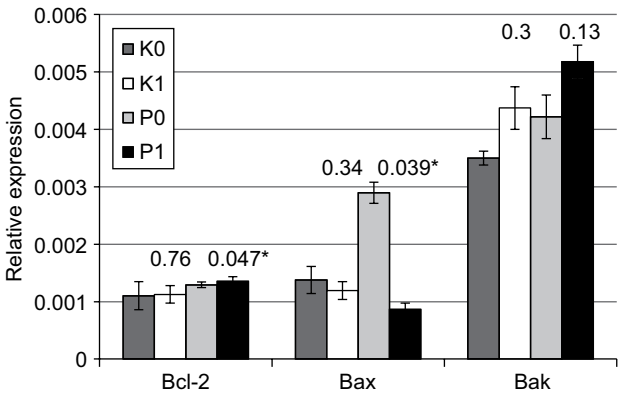

Figure 4. Relative expression levels of Bcl-2, Bax and Bak mRNA in K562 and PC-MDS cell line, treated by 1 μM 5-Aza for 24 h (K0=K562 control; K1=K562 1 μM 5-Aza for 24 h; P0=PC-MDS control; P1=PC-MDS 1 μM 5-Aza for 24 h). *p<0.05.

cells were treated with 1 μM 5-Aza for 24 h. Quantitative RT-PCR analyses of 10 genes were performed on total RNA extracted from both cell lines and from control untreated cells. The results of gene expression analyses are shown in Figures 2-5.

Expression analysis of the cyclin-dependent kinase inhibitor p15/INK4b in 5-Aza-treated PC-MDS cells showed significant upregulation (re-expression) of this important tumor suppressor gene, upon demethylation therapy ($p<0.05$).

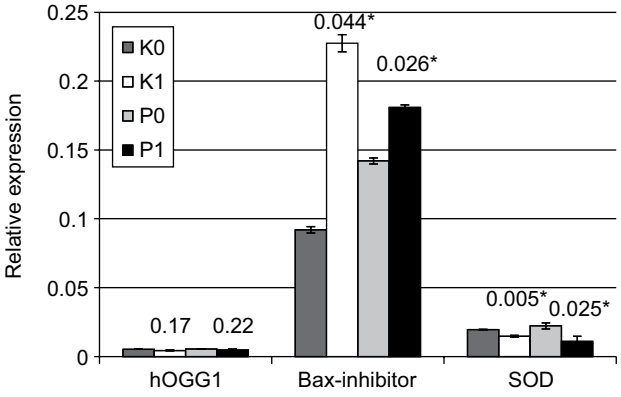

Figure 5. Relative expression levels of hOGG1, Bax-inhibitor and SOD mRNA in K562 and PC-MDS cell line, treated by 1 μM 5-Aza for 24 h (K0=K562 control; K1=K562 1 μM 5-Aza for 24 h; P0=PC-MDS control; P1=PC-MDS 1 μM 5-Aza for 24 h). *p<0.05.

Our results showed that 5-Aza inhibits the DNMT1 in both PC-MDS and K562 cell lines, and this downregulation was significant ($p<0.05$) in the MDS cell line (Figure 3). Similar result was obtained for the DNMT3A, whereas for the DNMT3B, the downregulation was significant in K562 cell line.

The results of mRNA expression for the genes involved in apoptosis regulation are presented in Figure 4. In both K562 and PC-MDS cell lines, mRNA of the antiapoptotic gene Bcl-2 was upregulated upon 5-Aza treatment, which was significantly different ($p<0.05$) from the untreated PC-MDS cells. The very interesting result of Bax downregulation, together with Bax-inhibitor significant ($p<0.05$) upregulation (Figures 4 and 5) may indicate that Bax, the proapoptotic gene, is not involved in the increased cytotoxicity of 5-Aza-treated cells. The mRNA of proapoptotic Bak gene was upregulated upon 5-Aza treatment in both cell lines, but this increase in mRNA expression levels was not statistically significant (Figure 4).

The downregulation of mRNA for 8-oxoguanine (8-oxoG) DNA glycosylase in 5-Aza-treated K562 and PC-MDS cells was not significant in comparison with untreated cells ($p<0.05$), whereas the mRNA of superoxide dismutase (SOD) gene was significantly downregulated ($p<0.05$) in 5-Aza-treated K562 and PC-MDS cell lines (Figure 5).

Discussion

Epigenetic therapy with single-agent azanucleoside hypomethylating drugs is effective in inducing remissions in patients with MDS and acute myeloid leukemia (AML), particularly when given at low doses [19,22]. Clinical trials addressing combination therapies of demethylating agents with multiple investigational agents as well as novel combination regimens are ongoing [23]. To this purpose, adequate *in vitro* models of MDS, such as our novel PC-MDS cell line, are needed.

The cytotoxic effects of 5-Aza in PC-MDS cell line are in accordance with the results of Khan et al. [24]. They showed that 24-h treatment of P39 myeloid cell line (derived from a patient with MDS-chronic myelomonocytic leukemia) with 1 μM 5-Aza induces apoptosis of < 30% of leukemic cells.

We found statistically significant downregulation of DNMT1 and DNMT3A in PC-MDS cell line. Our results are in accordance with the results obtained in breast cancer MCF-7 cells treated with 5-Aza-CdR, in which downregulation of DNA methyltransferase 3b mRNA was found. Xiong et al. [25] concluded that 5-Aza-CdR might retard the growth of tumor cells and promote apoptosis of MCF-7 breast cancer cells by inhibiting the expression of DNA methyltransferase 3b and re-activating the Apaf-1 gene expression. On the other hand, Patel et al. [26] performed quantitative RT-PCR of DNMT1 mRNA and showed that the reduction in DNMT1 protein at 24 h was not caused by reduced transcription, as it is the case in our results with PC-MDS cell line. Patel et al. demonstrated that 5-Aza-CdR induced degradation of preformed DNMT1 protein by pre-treating cells with the protein synthesis inhibitor cycloheximide prior to exposure to 5-Aza-CdR [26]. The downregulation of DNMT1 and DNMT3A upon 5-Aza treatment was statistically significant only in the PC-MDS cell line, indicating cell-type specific differences in response to 5-Aza treatment between PC-MDS and K562 cells.

The major mutagenic base lesion in DNA caused by exposure to reactive oxygen species (ROS) is 8-oxoG. This damaged base is excised by a DNA glycosylase with an associated apurinic, apyrimidinic lyase activity for chain cleavage. Enzymatic mechanisms are important in the defence from ROS damage, and among them, DNA repair plays a major role [27]. The induction of DNA damage by decitabine (and, presumably, also by 5-Aza), combined with a role of DNMT1 in DNA repair, indicates that drug-induced demethylation patterns might be influenced by DNA repair mechanisms [28]. Our results of non-significant downregulation of 8-oxoG DNA glycosylase (hOGG1) gene that encodes a major DNA glycosylase for oxidized lesions [29], may indicate that 5-Aza treatment decreases the cellular ability to repair the DNA damage. Our results may suggest that 5-Aza induces cytotoxicity due to strand breaks, owing to 5-Aza potential to inhibit DNA-repair mediated by hOGG1.

Abundant examples exist that the regulation of

genes encoding either antiapoptotic or proapoptotic Bcl-2- family proteins is altered in cancers [30]. Yip et al. showed in a multiple myeloma cell line, that upon 5-Aza treatment (2.5 μmol/L for 0, 16, 24 and 36 h) no significant changes were detected in the expression levels of the antiapoptotic Bcl-2 and Bcl-xl proteins, but there was a significant increase in the proapoptotic family members Bax, Noxa, and Puma-α, which peaked at 24 h [30]. Our results showed that the moderate induction of apoptosis in PC-MDS cells (Figure 1; 76.5% of cells remained viable), treated by 1 micro mole of 5-Aza, relied solely on upregulation of Bak mRNA (coding the pro-apoptotic protein), whereas the other modifications of gene expression concerned the anti-apoptotic genes (downregulation of Bax and upregulation of Bax-inhibitor and Bcl-2). The comparative analysis of K562 and PC-MDS cell lines showed that the differences in Bcl-2 and Bax gene expression between 5-Aza-treated and non-treated cells were statistically significant only in the PC-MDS cell line.

Target genes being particularly prone to demethylation by demethylating drugs in aberrant cells (e.g. p15/INK4b) are under active investigation. Therefore, most studies in this context have focused on the p15 tumor suppressor gene, which can be hypermethylated in MDS and AML patients and can be demethylated and reactivated in patients undergoing azacytidine and decitabine therapy [29]. Our results of p15 mRNA overexpression in PC-MDS cell line upon 5-Aza treatment are in accordance with the results of Berg et al. showing demethylation of a hypermethylated p15 gene, as well as p15 protein overexpression in acute myeloid leukemia cells, upon DNMT inhibitor treatment [31]. It is of particular interest to emphasize the difference in response between K562 and PC-MDS cell lines, regarding the p15 gene upregulation, showing statistically significant p15 re-expression upon 5-Aza treatment only in the PC-MDS cell line.

Oxidative stress can contribute to DNA lesions, which interfere with the ability of DNA to function as a substrate for the DNMTs, resulting in global hypomethylation, frequently occurring early in the progression phase of neoplasia [12]. SODs are essential enzymes responsible for the elimination of superoxide radicals by converting them into hydrogen peroxide (H_2O_2), which is then further converted to non-harmful substances (O_2, H_2O) by enzymes such as catalases and peroxidases. Our results indicate that the 24h treatment of PC-MDS and K562 cell lines by 1 μM of 5-Aza, inhibits SOD, and according to results of Gao et al., presumably cause an accumulation of cellular superoxide (O_2^-), and damage to mitochondrial membranes, leading to release of cytochrome c and activation of apoptosis in acute myeloid leukemia cells [32]. Our results of SOD downregulation upon 5-Aza treatment, which increases the intracellular ROS levels, are also in accordance with results of many authors (reviewed by Pelicano et al. [33]), showing that the drug-induced increase in superoxide inhibits SOD expression. Madesh et al. [34] showed that the enhancement of superoxide production in hepatocytes triggers the Bak's proapoptotic activity in a Bax-independent fashion. According to our data and the fact that our methodological approach was directed towards the cytotoxicity assessment, without the possibility to distinguish the exact type of cell death, we may hypothesize that the significant upregulation of Bak stimulates the 5-Aza-induced cytotoxicity in PC-MDS cells, and opposes the anti-cytotoxic effects induced by the upregulation of Bcl-2 and Bax inhibitor.

The details of the molecular pathways and networks involved in 5-Aza cytotoxicity are beyond the scope of this paper, aiming to further characterize the PC-MDS cell line. Therefore, large-scale experiments are needed to elucidate the molecular mechanisms underlying the 5-Aza effects in MDS cells.

Conclusion

According to our preliminary data, we may conclude that the 5-Aza treatment of PC-MDS cells induced significant differences in the expression of the genes involved in DNA methylation, apoptosis and antioxidative defence, in comparison with non-treated PC-MDS cells. Evaluation of our qRT-PCR results showed that the re-expression of p15 in the PC-MDS cell line implicates the possibility that this novel cell line may be a suitable model for the studies of pharmacologic demethylation as a possible mechanism resulting in hematologic response in MDS.

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