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Azacytidine Enhances Efficacy of Tyrosine Kinase Inhibitors in Aml Cells

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ABSTRACT

Background: Azacytidine **(5-Aza)** is a chemotherapeutic drug used for DNA-de-methylation resulting in re-expression of silenced tumor suppressor genes **(TSG)**. Epigenetic silencing of TSG is mediated by DNA methylatransferases **(DNMTs)** in several tumors and has been reported to play important roles in leukemogenesis. Cancer formation could be avoided by inhibition of DNMTs and over expression of TSG. Re-expression of TSG such as *SHP-1* and *SOCS-3* is inversely proportionate with STAT3 signaling.

Lestaurtinib **(CEP-701)** and midstaurin **(PKC-412)** are multi-targeted tyrosine kinase inhibitors **(TKI)** that potently inhibit FLT3 tyrosine kinase and induces hematological remission in AML patients. However, resistance to both TKI is recorded in majority of AML patients in clinical trials. Therefore, the aim of this study, was to assess the effect of 5-Aza on re-expression of TSG and sensitivity response to CEP-701 and PKC-412 in resistant AML cells

Methods: Resistant cells were developed by overexposure of MV4-11 cells to CEP-701 and PKC-412, individually and treated with 5-Aza. Cytotoxicity of both TKI and apoptosis were determined using Annexin V and MTS assays, respectively. Gene expression profiling using microarray and

real time-PCR, STATs activity was examined using Western blot and methylation status of *SHP-1, SOCS-1, SOCS-3 and PRG2* was determined by methylation specific-PCR and pyrosequencing analysis.

Results: Cytotoxic doses of CEP-701 and PKC-412 on MV4-11cep+5-Aza and MV4-11pkc+5-Aza cells were significantly decreased compared with resistant cells. There was significant hypomethyaltion (p=0.002) in CPG islands of *SHP-1* and *PRG*, associated with their re-expressions. The higher sensitivity to TKI was associated with STAT3 inactivation in 5-Aza treated cells compared with their resistant cell lines.

Conclusion: 5-Aza enhances efficacy of TKI in AML cells and treatment with 5-Aza followed by PKC-412 or CEP-701 could provide suitable candidates for further investigates to underline alternative options for the treatment of AML patients with FLT3-ITD.

Keywords: AML; Azacytidine; Sensitivity; TSG; STAT3

INTRODUCTION

Leukemia is a hematological malignancy characterized by accumulation of malignant leukocytes in bone marrow and peripheral blood **(Hoff brand et al, 2011)**. It occurs due to serial mutational events such as amplification, point mutations and specific chromosomal translocations forming fusion genes resulting excessive proliferation and differentiation impairment of progenitor cells [1,2]. However, mutational events are not the only cause of leukemia or other cancers but also epigenetic silencing of tumor suppressor genes **(TSG)** has been reported to play important roles in leukemogenesis.

Epigenetic silencing due to hypermethylation of CPG islands is a frequent mechanism of inactivation of tumor suppressor genes **(TSG)** in a variety of human cancers including AML [3]. DNA methylation is an epigenetic modification that acts as regulators of gene expression [4]. DNA Methylatransferases Genes **(DNMT)** encode enzymes that are responsible for methylation by stimulating the transfer of methyl group to cytosine resulting in 5-methylcytosine [5]. In human cells, DNA methylation is caused by *DNMT1, DNMT3a* ***(DNMT3α)*** and *DNMT3b* ***(DNMT3β)*** [6-9].

Up-regulation of *DNMT1* and *DNMT3a* or *DNMT3b* has been recorded in several cancers; stomach cancer [10], breast cancer [11], pancreatic cancer [12], hepatic cancer [13], cervical cancer [14] and AML [15]. The over expression of *DNMT1* was associated with poor prognosis. However, repression of *DNMT1* expression results in inhibition of proliferation in cancer cells, in vitro and in vivo [16-19].

Azacytidine **(5-Aza)** and 5-Aza-2-deoxycytidine **(5-Aza2dc)** are strong inhibitors of *DNMTs* and used as chemotherapeutic drugs that induces DNA de-methylation and approved to be the standard care for patients with myelodysplastic syndromes **(MDS)** [20,21]. Treatment with 5-Aza and cytotoxic anticancer drugs exhibits synergistic activity in AML and NSCLC cells [22].

Fms-like tyrosine kinase-3 **(FLT3)** is a member of type III RTK that plays important roles in cell survival, proliferation, and differentiation during normal hematopoiesis [23]. Primarily, FLT3 is expressed on immature myeloid and some lymphoid hematopoietic progenitors cells [24,25] and in majority **(70-100%)** of AML and B-ALL patients [26,27]. Mutations of FLT3 are found in 30% of AML patients. These mutations include FLT3-ITD, that results in constitutive activation in many signaling pathways such as STAT, MAP kinase and AKT pathways [28-31]. Therefore, TK kinase such as FLT3 is important targets in the treatment of human cancers including AML.

Current treatment of AML relies on remission induction **(induction)** with cytosine arabinoside **(Ara-c)** and anthracycline followed by consolidation (post-remission therapy) with additional intensive chemotherapy or stem cell transplantation [32,33]. Most of AML patients **(50-90%)** relapse with negative complications due to chemotherapies resulting in a significant higher morbidity and mortality, particularly in elderly patients [28, 34-37]. Therefore, the development of alternative less toxic and more specific therapy for such patients is vitally needed. The success of tyrosine kinase inhibitors **(TKI)** in CML encourage searching for such small molecule inhibitors to treat AML patients. Subsequently, several TKI that potentially and specifically target tyrosine kinases such as FLT3, KIT and PDGF have been developed as alternative option for AML patients [38, 39].

CEP-701 and PKC-412 are TKI that specifically target FLT3 and showed advancement in clinical trials for AML patients [40,41]. However, acquired resistance to these TKI is reported in majority of AML patients in third phase of clinical trials [40,42]. JAK/STAT pathway is one of signaling network that plays critical role in various cell biological activities including immune response, cell growth and differentiation [43-45]. Normally, JAK/STAT pathway transmits signals to hematopoietic stem cells in bone marrow for hematopoiesis in response to stimuli such as erythropoietin **(EPO)**, thrombopoietin **(TPO)**, growth hormone and granulocyte-macrophage colony-stimulating factor **(GM-CSF)** [46-48]. In addition, JAK/STAT signaling regulates critical cellular proliferation, differentiation and apoptosis [49]. Moreover, in normal cells, STATs proteins are activated by receptor-associated JAK kinases resulting in nucleus translocation for transcription. Hence, the STATs phosphorylation and progression of signals transduction are JAK dependent [50].

However, deregulations of JAK/STAT signaling were reported in many cancers including hematological malignancies [51-54]. FLT3 mutations cause constitutive activation of STATs that enhances leukemogenesis [55-57]. Additionally, FLT3-ITD mutations are associated with poor prognosis [58]. The activated signaling pathways result in uncontrolled proliferation, impairment of differentiation and enhance cell survival [27]. Furthermore, the activation of STAT signaling pathways plays roles in development of resistance to TKI such as ABT-869 [59]. On the other hand, the inhibition of JAK/STAT signaling results in suppression of cells proliferation and induces apoptosis in various cancer [60-62].

JAK/STAT signaling is negatively regulated by; protein tyrosine phosphates **(PTP)** such as SHP-1 gene, suppressors of cytokine signaling **(SOCS)** and protein inhibitors of activated STATs **(PIAS)** [63-67]. However, JAK/STAT negative-regulators have been shown to have low activity due to epigenetic silencing in cancers [68]. Additionally, JAK/STAT signaling is frequently activated in several cancers including AML due to methylation of *SHP-1* promoter region [69]. *SHP-1, SOCS-1* and *SOCS-3* function as TSG [70-72]. The TSG have been shown to be inactivated due to aberrant methylation in leukemia, lymphoma and multiple myeloma [73,74]. Moreover, over expression of bone marrow proteoglycan 2 ***(PRG2)*** was absent in methylated cancer cells and its re-expression by 5-Aza2dc induced apoptosis in pancreatic cancer cell lines [75].

In this study it was hypothesized that, inhibition of DNMT by 5-Aza de-methylating agent could enhance TSG re-expression and confer sensitivity responses to TKI in myeloid resistant cells. Therefore, gene expression profiling, methylation analysis and cytotoxicity with apoptosis assays were studied before and after treatment of resistant cells with 5-Aza.

MATERIALS AND METHODS

Lestaurtinib **(CEP-701)** and Midostaurin **(PKC-412)** were purchased from LC Laboratories **(Woburn, MA, USA)** and dissolved in DMSO before use. The stock preparation was 1 mM for each TKI, which was stored at −20˚C according to the manufacturer's protocol.

Development of Resistant Cells

AML cell line with *FLT3*-ITD **(MV4-11)** was obtained from Department of Hematology, UniversitiSains Malaysia **(USM)**, having originally been purchased from American Type Culture Collection **(ATCC)**. The cells were cultured with RPMI 1640 **(Life Technologies, Grand Island, NY, USA)** supplemented with 10% fetal bovine serum **(FBS; Life Technologies, Grand Island, NY, USA)** at a density of 5 × 104 cells/mL in a humid incubator with 5% CO2 at 37˚C. Resistant cell line to CEP-701 and PKC-412, namely MV4-11R-cep and MV4-11R-pkc, respectively were developed according to the protocol described previously [76]. Briefly, log phase growing MV4-11 cells were co-cultured at a starting dose of 20 nM TKI followed by a step-wise increase in concentration of 10–20 nM for 12 months until the cells were able to survive at the IC_{50} dose of each TKI on parental MV4-11. The resistant cell lines were grown in normal medium without TKI for at least 48 h before starting the experiments.

Azacytidine Treatment

Azacytidine **(5-Aza; Sigma-Aldrich Corp, MO, USA)** was dissolved by injecting the vial of 5-Aza (1.2 mg) with 10 ml RPMI-1640 media and the stock concentration was 500 μM for use immediately or stored at -20˚C to be used within 2-3 days. Five μM was prepared as working solution by adding 1 ml 5-Aza stock preparation to 99 ml complete media. Resistant cells were sub-cultured in working solution (5 μM) and incubated in a humid incubator with 5% CO2 at 37˚C for 4-5 days until confluent. The resistant cells that treated with 5-Aza were designed as

MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells and sub-cultured in normal media without treatment for at least one passage before re-treatment with CEP-701 or PKC-412.

Growth Inhibition Assay

MV4-11 cells were seeded in 96-well culture plates at a density of 1×10^4 viable cells/100 μL/well in triplicates, and were treated with CEP-701 and PKC-412 individually. Colorimetric Cell Titer 96 Aqueous One Solution Cell Proliferation assay **(MTS Assay; Promega, Madison, WI, USA)** was used to determine the cytotoxicity's of each TKI. The IC_{50} values were calculated using Graph Pad Prism 3.02 **(San Diego, California, USA)**. Each experiment was performed in triplicate.

Apoptosis Assay

Annexin V–FITC binding assay **(BD Pharmingen, San Diego, California, USA)** was used as recommended by the manufacturer and analyzed by flowcytometry **(BD FACSCanto™, San Jose, California, USA)**. Analysis was performed with Diva software **(FACS Diva, 6.1.2, San Jose, California, USA)**. Each experiment was performed in triplicate.

RNA Extraction

Total RNA was extracted from all cell lines **[MV4-11, MV4-11R-cep, MV4-11R-cep+5-Aza, MV4-11R-pkc and MV4-11R-pkc+5-Aza]** using the Rneasy® Mini Kit **(Qiagen, Valencia, California, USA)**, the purity and concentration was measured with a NanoDrop ND-1000 spectrophotometer V3.3.0 (**NanoDrop Technologies, Berlin, Germany)**.

Gene Expression Profiling

The Prime View™ Human Gene Chip Arrays were used for gene expression profiling according to the manufacturer's protocol (Affymetrix, Santa Clara, California, USA). Briefly, first-strand cDNA was synthesized from total RNA and the cDNA was converted into a double-stranded DNA template for transcription.

Scanning and data analysis

The Gene Chip arrays were scanned using Gene Chip® Scanner 3000 **(Affymetrix)** and the analysis was performed using Agilent's Gene Spring GX software 12.1. One way ANOVA was conducted and two-fold change used as criteria to determine the expression. The data were further analyzed by Functional Annotation Tool DAVID Bioinformatics Resources 6.7, NIAID/NIH **(http://david.abcc.ncifcrf.gov)**. JAK/STAT family members and genes involved in methylation were our interest in this study. Therefore, KEGG_pathway functional annotation table and gene ontology **(GO)** annotations related to methylation were studied. After notification that, *PRG2* gene showed the highest fold changes in all 5-Aza treated cells, GO related to carbohydrate binding were added to the interested genes to look for the expression.

Quantitative Real Time-PCR (RQ-PCR)

High Capacity RNA-to-cDNA kit **(Applied Biosystem, Foster City, California, USA)** was used to synthesize cDNA according to the manufacturer's protocol. TaqMan Gene Expression assays **(Applied Biosystems)** were performed on an Applied Biosystem 7500 Fast Real-Time PCR System according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase ***(GAPDH)*** was used as an internal control. ABI 7500 software v2.0.6 (Applied Biosystem) was used to validated the expression of 4 interested genes, SHP-1,SOCS-1, SOCS-3 and *PRG2* in all cell lines using the comparative threshold cycle **(Ct)** method.

DNA Extraction

DNA was extracted from all cell lines using the NucleoSpin® Tissue kit **(Macherey-Nagel, Düren, Germany)** following the manufacturer's instructions. The concentration and purity of DNA were measured by NanoDrop.

METHYLATION-SPECIFIC POLYMERASE CHAIN REACTIONS (MS-PCR)

One microgram of DNA was treated with bisulfate using the EZ DNA Methylation-Gold™ Kit **(Zymo Research, Irvine, NY, USA)** according to the manufacturer's instructions. MS-PCR was performed as described previously [77] and modified DNA was subjected to two separate PCRs. MS-PCR primers were designed to amplify the methylated (M) or un methylated **(U)** alleles. SHP-1 **(Gene Bank: NM_002831)** was amplified using previous designed primers [78]. Universal methylated DNA **(Zymo Research, Irvine, NY, USA)** was used as a positive control. The 50-μL PCR reaction contained 200 ng of bisulfate-treated DNA, Reddy Mix PCR master mix **(Bioline Ltd., London, UK)** and 0.2 μM of each primer. PCRs were performed in a thermal cycler (**PTC-200, Alameda, California, USA).** The amplified PCR products were denatured for 2 min at 95˚C followed by 40 cycles: 95˚C for 25 s, 59˚C for 35 s, 52˚C and 72˚C for 65 s, and extension at 72˚C for 5 min. PCR products were electrophoresis on 2% agar's gels, and visualized by ethidium bromide staining under ultraviolet transillumination. Results from triplicate experiments were used to determine methylation status.

Pyrosequencing Analysis

As mentioned in published work [79, 80], 20 μL (1 μg) of purified DNA from each sample were sent to EpigenDx **(Hopkinton, MA, USA)** for pyrosequencing analysis of *SHP-1* and *PRG2* genes after 5-Aza treatment. The assays were designed to target 6 and 4 CPG islands in the promoter regions of the *SHP-1* and *PRG2* genes, respectively.

Western Blot Analysis

Protein from all cell lines was extracted by RIPA buffer (Sigma-Aldrich, MO, USA), described in published work MV4-11, MV4-11R-cp, MV4-11R-cep+5-Aza, MV4-11R-pkc and MV4-11R-

pkc+5-Aza cell lines were incubated with 300 nM CEP-701 and PKC-412, accordingly for 3 days before protein extraction. Bio-Rad protein dye **(Bio-Rad, Hercules, California, USA)** and a spectrophotometer **(BioPhotometer Plus, Eppendorf, Germany)** were employed for the measurement of protein concentrations. Preparation of immunoblotting was performed as described previously [81]. Antibodies used were anti-STAT1, anti-p-STAT1, anti-STAT3, anti-p-STAT3, anti-STAT5, anti-p-STAT5, and anti-β-actin **(Thermo Scientific, Waltham, MA, USA)**.

Statistical and Bioinformatics Analysis

Repeated-measures ANOVA and Kruskal–Wallis tests were employed for statistical analyses. All statistical analyses were performed using the SPSS software package **(Version 20, SPSS, and Armonk, NY, USA)** and a p value <0.05 was considered as significant. For microarray results analysis, Agilent's GeneSpring GX software 12.1 was used and one way ANOVA test was applied and a minimum 2 fold change was used as criteria to determine the expression. Further analysis was performed using DAVID Functional Annotation Bioinformatics Microarray Analysis 6.7, NIAID/NIH. The modified Fisher Exact P-value was generated from DAVID Functional Annotation Bioinformatics Microarray Analysis and the adjusted p value ≤ 0.1 was considered as significant.

Results

Higher sensitivity to CEP-701 and PKC-412 in 5-Aza treated cells

The MTS assay showed a significant decrease in the IC50 of CEP-701 **(p= 0.002)** and PKC-412 **(p= 0.003)** on MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells, respectively compared to other cells (Figure 1a and b).

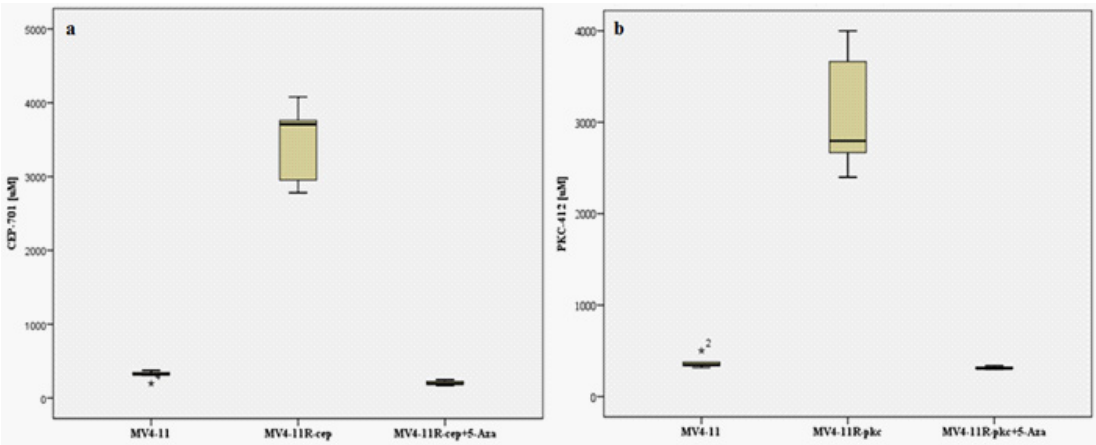

Figure 1: Cell growth inhibition by CEP-701 and PKC-412 on parental and resistant cells before and after 5-Aza treatment. a) Depicts the IC50 of CEP-701 on MV4-11, MV4-11R-cep and MV4-11R-cep+5-Aza cell lines. MV4-11R-cep cells show a significant higher cytotoxic dose of CEP-701 compared to other cells (p=0.002). However, MV4-11R-cep+5-Aza cells display the lowest cytotoxic dose. There is no significant difference in the cytotoxic dose of the drug

between MV4-11 and 5-Aza treated cells (p=0.066). b) The IC50 of PKC-412 on MV4-11, MV4-11R-pkc and MV4-11R-pkc+5-Aza cells. The cytotoxic dose of PKC-412 on MV4-11R-pkc cells shows a significant higher compared with MV4-11 and MV4-11R-pkc+5-Aza cells (p=0.003). However, there is no significant difference in the IC50 between MV4-11 and MV4-11R-pkc+5-Aza cells. The outlier value marked as *2.

However, the resistant cells displayed the highest cytotoxic doses of CEP-701 and PKC-412.

Higher Apoptosis in 5-Aza Treated Cells

The results showed a significant increase in the apoptosis in MV4-11R-cep+5-Aza cells compared to MV4-11 and MV4-11R-cep cells based on CEP-701 concentration **(F= 23.48, p= <0.001)** (Table 1). In contrast, the results showed a significant increase in the apoptosis in MV4-11R-pkc+5-Aza cells compared to other cells based on PKC-412 concentration **(F= 33.55, p= <0.001)** (Table 2).

Table 1: Comparison of mean apoptotic cells % in cells based on CEP-701 concentration.

CEP-701 **concentration [nM]**	**Cell lines**	**Mean percentage of apoptotic cells**	**95% CI**
100	MV4-11 MV4-11R-cep MV4-11R-cep+5-Aza	13.51 10.50 26.03	11.77, 15.25 8.59, 12.41 23.33, 28.72
200	MV4-11 MV4-11R-cep MV4-11R-cep+5-Aza	32.07 15.19 45.70	29.27, 34.86 12.13, 18.25 41.37, 50.03
300	MV4-11 MV4-11R-cep MV4-11R-cep+5-Aza	56.46 22.85 64.55	51.06, 61.86 16.93, 28.77 56.18, 72.92

Repeated measure ANOVA between group analysis with regard to concentration was applied. Assumptions of normality, homogeneity and compound symmetry were checked and were fulfilled.

There was a significant difference of mean percentages of apoptotic cells among three cell lines based on CEP-701 concentration (F = 23.48, p<0.001). MV4-11R-cep+5-Aza cells show the highest apoptosis at all drug concentrations. However, MV4-11R-cep cell lines display the lowest apoptosis.

Table 2: Comparison of mean apoptotic cells % in cells based on PKC-412 concentration.

PKC-412 concentration [nM]	Cell lines	Mean percentage of apoptotic cells	95% CI
100	MV4-11 MV4-11R-pkc MV4-11R-pkc+5-Aza	11.67 3.75 15.30	10.849, 12.486 2.931, 4.569 14.481, 16.119
200	MV4-11 MV4-11R-pkc MV4-11R-pkc+5-Aza	18.87 7.53 26.03	17.197, 20.538 5.855, 9.195 24.362, 27.703
300	MV4-11 MV4-11R-pkc MV4-11R-pkc+5-Aza	47.07 13.10 58.77	45.007, 49.128 11.039, 15.161 56.707, 60.828

There was a significant difference of mean percentages of apoptotic cells among three cell lines based on PKC-412 concentration ($F = 33.55$, $p<0.001$). MV4-11R-cep+5-Aza cells show the highest apoptosis at all drug concentrations. The highest apoptosis was seen in 5-Aza treated cells at all drug concentrations. However, the resistant cell lines display the lowest apoptosis.

RESTORATION OF SHP-1 AND PRG2 GENES EXPRESSION IN 5-AZA TREATED CELLS

To investigate the correlation between re-expression of *TSG* and de-methylation, gene expression profiling using microarray was performed followed by RQ-PCR.

Microarray results revealed 1987 of 7227 **(27.5%)** genes and 1693 of 6382 **(26.5%)** genes involved in KEGG_pathway and demonstrated down-regulation in MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells, respectively compared to their resistant, MV4-11R-cep and MV4-11R-pkc cells. Interestingly, 41 genes of these genes are involved in JAK/STAT signaling and showed down-regulation, except *SHP-1* was up-regulated in both MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells compared to their resistant MV4-11R-cep and MV4-11R-pkc cells, respectively (Figures 2a and b).

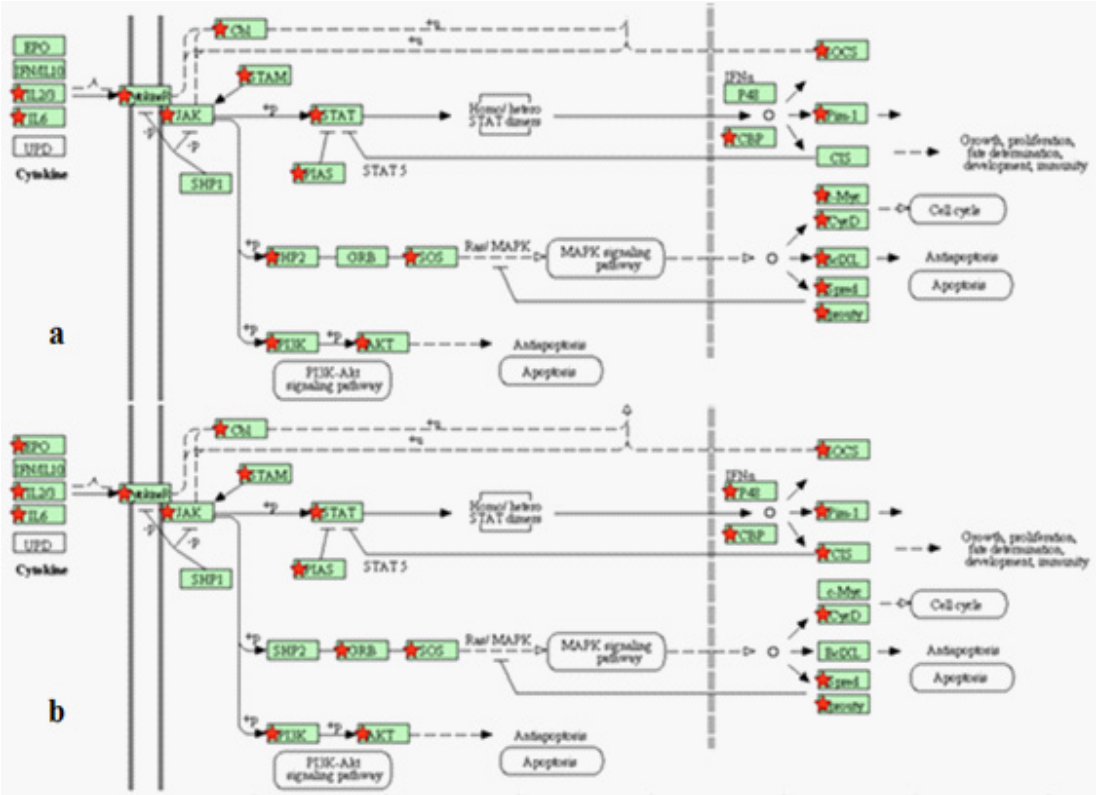

Figure 2: JAK-STAT signaling diagram in MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells. It displays down-regulation of most genes in JAK/STAT signaling (red stars) in; a) MV4-11R-cep+5-Aza cells and b) MV4-11R-pkc+5-Aza cells compared to MV4-11R-cep and MV4-11R-pkc cells, respectively. However, SHP-1 and few other genes are not down-regulated in both 5-Aza treated cells, by DAVID online software (http://david.abcc.ncifcrf.gov/).

On the other hand, GO annotation revealed a significant up-regulation of *PRG2* gene with the highest fold changes, 93 and 114.55 times higher in MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells, respectively compared to other cells(Table 3).Furthermore, to validate the re-expression of *SHP-1* and *PRG2*, RQ-PCR was performed and the results showed significant up-regulation of both genes in MV4-11R-cep +5-Aza cells and MV4-11R-pkc+5-Aza cells compared to other cells, published work [79, 80] and Figure 3a and b.

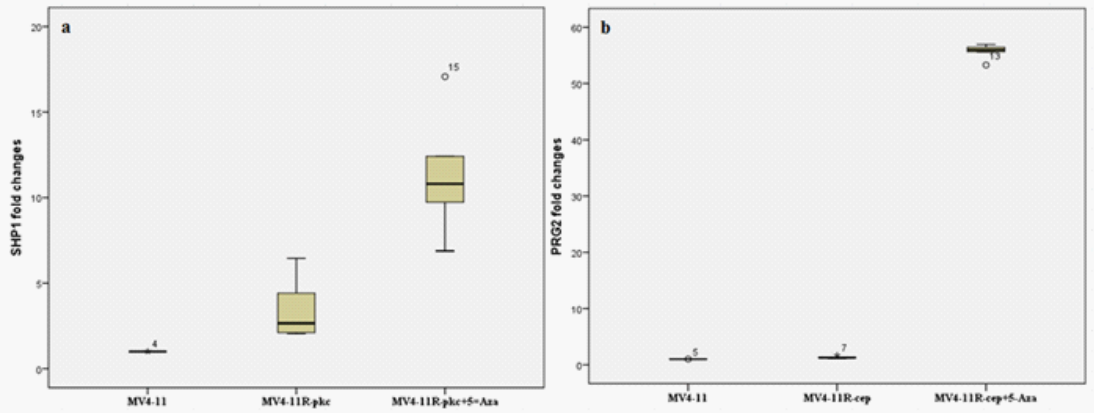

Figure 3: Depicts results of RQ-PCR in parental, resistant and 5-Aza treated cells. The results revealed marked re-expression of SHP-1 and PRG2 genes in 5-Aza treated cells compared with other cells.KruskalWllis Test was applied followed by Multiple Mann-Whitney Test with Bonferruni correction. a) SHP-1shows a significant re-expression in MV4-11R-pkc+5-Aza compared to MV4-11 and MV4-11R-pkc cells (p=0.001).b) PRG2re-expressed with the highest fold changes (56 times higher) in MV4-11R-cep+5-Aza cells compared to MV4-11 and MV4-11R-cep cells (p=0.002).

INHIBITION OF DNA METHYLTRANSFERASE IN 5-AZA TREATED CELLS

Gene Ontology also showed 46 and 50 genes display a significant down-regulation (p<0.001) in MV4-11R-cep+5-Aza cells compared with MV4-11 and MV4-11R-cep cells, respectively and are involved in methylation (data not shown), including DNMTS **(DNMT1, DNMT3a and DNMT3b)**, Table 3. The results also revealed significant down-regulation of DNMT3a and DNMT3b (p<0.001) in MV4-11R-pkc+5-Aza compared with MV4-11R-pkc cells (Table 3).

Table 3: Down-regulation of DNMTs and up-regulation of PRG2 in MV4-11R-pkc+5-Aza cells.

Comparison	ID	Gene Symbol	Regulation	Fold Change
MV4-11R-cep+5Aza **VS** MV4-11R-cep	1786	DNMT1	down-regulated	10
	1788	DNMT3a	down-regulated	4.87
	1789	DNMT3b	down-regulated	28.98
	5553	*PRG2*	up-regulated	93
MV4-11R-pkc+5Aza **VS** MV4-11R-pkc	1788	DNMT3a	down-regulated	2.35
	1789	DNMT3b	down-regulated	23.29
	5553	*PRG2*	up-regulated	114.55

Low Methylation of SHP-1 and PRG2 Gene in 5-Aza Treated Cells

The results of MS-PCR showed partial methylation of *SOCS-1, SOCS-3* and *SHP-1* in MV4-11 and resistant, MV4-11R-cep and MV4-11R-pkc cells **(data not shown)**. However, after treatment with 5-Aza, only *SHP-1* and *PRG2* were re-expressed markedly. Therefore, further analysis using pyrosequencing was applied for re-expressed genes and revealed higher methylation of the CPG islands in the promoter region of *SHP-1* and *PRG2* genes in parental and resistant cells. However, there was a significant lower methylation of CPG islands of both genes after treatment of resistant, MV4-11R-cep and MV4-11R-pkc cells with 5-Aza (Table 4).

Table 4: Percentage of methylation of CpG islands in the promoter region of *SHP-1* gene.

Sample ID	CpG-11	CpG -10	CpG -9	CpG -8	CpG -7	CpG-6	Mean	Min	Max
MV4-11	22.4	48.8	74.0	59.9	51.6	44.5	50.2	22.4	74.0
MV4-11R-cep	11.2	19.9	56.1	53.7	41.5	37.9	36.7	11.2	56.1
5-Aza treated cells	4.9	4.2	6.1	6.2	5.5	1.5	4.7	1.5	6.2
MV4-11R-pkc	36.5	34.0	56.6	42.7	38.8	30.7	39.9	30.7	56.6
5-Aza treated cells	4.7	12.0	14.2	13.1	13.5	8.4	11.0	4.7	14.2
Low Meth Control	6.8	6.9	2.7	10.5	8.3	5.9	6.8	2.7	10.5
Med Meth Control	52.2	53.0	52.1	42.7	48.4	49.1	49.6	42.7	53.0
High Meth Control	93.7	94.0	92.5	74.5	83.3	93.8	88.6	74.5	94.0

Pyrosequencing analysis showing methylation levels of 6 CpG islands of *SHP-1* gene. It demonstrates low methylation in CpG islands of *SHP-1* in 5-Aza treated cells compared with other cells.

The results showed no significant difference in methylation levels of CPG islands in the promoter region of *SHP-1* and *PRG2* gene in MV4-11 compared with MV4-11R-cep+5-Aza cells and MV4-11R-pkc+5-Aza cells (Figure 4) and published work [79, 80].

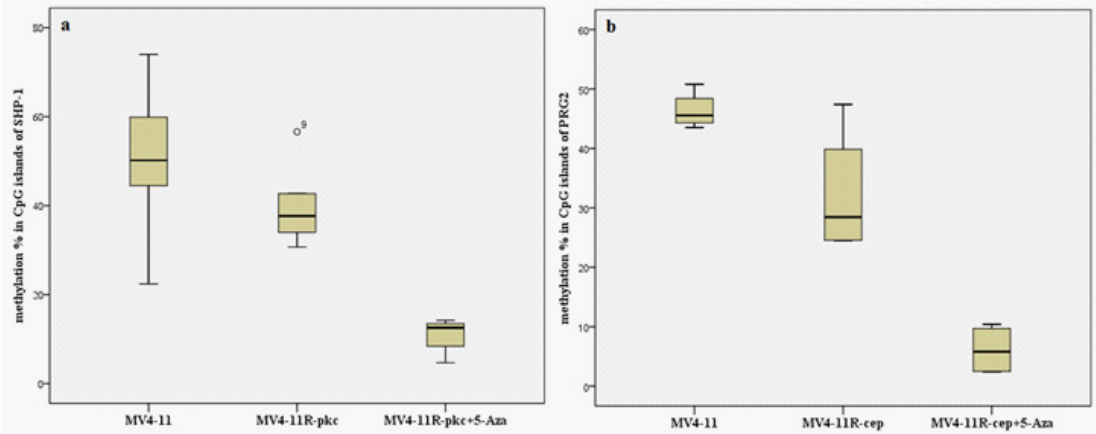

Figure 4: Low methylation of SHP-1 and PRG2in MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells.Pyrosequencing analysis describesmethylation levels of CpG islands in the promoter region of SHP-1and PRG2. Kruskal–Wallis test was applied followed by the Multiple Mann–Whitney Test with Bonferroni correction. The box blot shows significant lower of methylation in CpG islands of a)SHP-1(p=0.002) inMV4-11R-pkc+5-Aza cells and, b)PRG2(p=0.015)inMV4-11R-cep+5-Aza cells, respectively compared with other cells.However, there was no significant difference in the methylation levels of CpG islands of SHP-1(p=0.150) and PRG2(p=0.149)in MV4-11 cells compared with MV4-11R-cep cells.

STAT3 Inactivation in 5-Aza Treated Cells

In published work, [79, 80] it was found that only STAT3 activated in resistant cells. However, it was inactivated after treatment of resistant cells with 5-Aza.

DISCUSSION

Azacytidine **(5-Aza)** is a strong inhibitor of *DNMT* that has been known to restore the expression of TSG by de-methylation and shown clinical efficacy in MDS [82-84]. It is an important option in the treatment of MDS/AML patients [85] and currently being used in UK for the treatment of some adults with MDS, CML and AML [86]. Initial results from a phase 1/2 study of combination of PKC-412 and 5-Aza in refractory or relapsed AML reveals good response with a complete remission rate of 25% and 20% of patients achieving complete remission with incomplete platelet recovery [87]. CEP-701 and PKC-412 are the most intensively studied TKI that have shown advancement in clinical trials of AML patients [40, 41]. However, acquired resistance to CEP-701 and PKC-412 has been documented *in vitro* and *in vivo* [40, 42, 59, 88-91]. Therefore, this study aimed to assess the effect of 5-Aza on re-expression of TSG and sensitivity to TKI in resistant AML cells.

Tumor formation could be avoided by inhibition of *DNMT* due to hypo-methylation and reactivation of silenced TSG [92-94]. The tumor suppressor genes have been shown to be inactivated due to aberrant methylation in leukemia, lymphoma and multiple myeloma [73, 74].

DNMT1 is one of the target genes in cancer therapy because suppression of *DNMT1* results in de-methylation and re-expression of TSG [12, 95].

Epigenetic silencing of TSG is mediated by DNA methylatransferases (DNMT) in several tumors [6-9]. Up-regulation of *DNMT* has been recorded in several cancers, stomach [10], breast [11], pancreatic cancer [12], hepatic cancer [13], cervical cancer [14] and AML [15]. In accordance, the results of the present study indicate marked expression of DNMT in MV4-11, MV4-11R-cep and MV4-11R-pkc cell lines. The findings also revealed association of DNMT expression with hyper-methylation of *SHP-1, SOCS-1, SOCS-3* and *PRG2* genes in these cell lines. These findings are in agreement with previous reports in which, up-regulation of DNMT1 or DNMT3b results in transcriptional silencing of TSG due to hypermethylation [96-98]. Over expression of DNMTs could play roles in the pathogenesis of leukemia through aberrant hypermethylation [99].

In the published work, STAT3 showed activation in MV4-11R-cep and MV4-11R-pkc resistant cells in association with transcriptional silencing of *SHP-1, SOCS-1, SOCS-3* and *PRG2* [79, 80], suggesting critical roles of epigenetic silencing of these TSG in the activation of STAT3. This is consistent with previous reports in which, epigenetic silencing of *SOCS-1, SOCS-2* and *SOCS-3* is involved in the activation of STAT signaling **(Zhou et al., 2009)**. Transcriptional silencing of one component of JAK/STAT negative regulators is sufficient for activation of STAT signaling **(Johan et al., 2005)** and epigenetic methylation of *SHP1* contributes in the constitutive activation of STAT3 [100]. In addition, low level of *SHP-1* is not sufficient to inhibit activated STAT3 [101] and transcriptional silencing of *SHP-1* contributes in development of resistance to imatinib in *BCR-ABL1*-positive CML cells [102]. Moreover, the activation of STAT3 is an essential mechanism of imatinib resistance Bewry, et al. [103].

In similarity, over expression of *PRG2* in myeloid cells blocked G-CSF-dependent proliferation and increased apoptosis [104]. However, epigenetic silencing of *PRG2* is associated with higher proliferation and lowered apoptosis in pancreatic cancer cells [105] and leukemic cells [80]. Therefore, it could be suggested that, up-regulated DNMT in MV4-11R-cep and MV4-11R-pkc cells methylate *SOCS-1*, *SOCS-3*, *SHP-1* and *PRG2* genes resulting in their transcriptional silencing and activation of STAT3.

On the other hand, after treatment of resistant cells with 5-Aza, there was a significant down-regulation ($p<0.001$) of DNMTs in 5-Aza treated cells compared to untreated MV4-11, MV4-11R-cep and MV4-11R-pkc cells. These findings were consistent with that previously reported, OCI-AML3 and resistant CML (K562-R) cell lines treated with 5-Aza or 5-Aza2dc result in marked down-regulation of DNMTs [86,106]. Additionally, 5-Aza2c induced hypo-methylation in AML cell lines and patient blasts with re-expression of p15INK4b [107].

In similarity, the results of this study revealed higher sensitivity response to TKI after treatment of resistant cells with 5-Aza. These findings are in accordance with that reported by Nishioka, et al. [106], 5-Aza increases sensitivity response to dasatinib and nilotinib in resistant K562-R.

In the present study, gene expression results revealed also a significant re-expression of SHP-1 and PRG2 in MV4-11R-cep+5-Aza and MV4-11R-pkc+5-Aza cells that was associated with higher apoptosis and lower proliferation. These findings were in agreement with previous findings in which, re-expression of *SHP-1, SOCS-1, SOCS-2* and *SOCS-3* is associated with higher apoptosis and lower proliferation after treatment resistant cell with a de-methylating agent [59, 90]. Moreover, knocking down of DNMT1and DNMT3b resulted in re-expression of TSG and growth inhibition of cells with higher apoptosis in several cancers [12, 18, 19, and 96].

Furthermore, restoration of expression of *SHP-1* and *PRG2* was associated with STAT3 inactivation and apoptosis induction with higher sensitivity to TKIs; these findings are supported by that documented by Witzig, et al. [30], re-expression of SHP-1 results in inactivation of STAT3in diffuse large B cell lymphoma. Over expression of PRG2 inhibits cells proliferation and induces apoptosis [104].The inactivation of STAT3 induces apoptosis and reverts sensitivity to TKIs [103, 108].

Collectively, up-regulation of DNMT could play critical role in the acquisition of resistance to TKI through methylation of TSG resulting in inhibition of suppressor functions and activation of STAT3. However, repression of DNMT by 5-Aza reverts higher sensitivity response to TKI and re-expression of *SHP-1* and *PRG2* genes with inhibition of STAT3, Table 5.

Table 5: Percentage of methylation of CpG islands in the promoter region of *PRG2* gene.

Sample ID	CpG -6	CpG -5	CpG -4	CpG -3	Mean	Min	Max
MV4-11	50.8	46	45.1	43.5	46.3	43.5	50.8
MV4-11R-cep	24.5	24.6	32.3	47.4	32.2	24.5	47.4
MV4-11Rcep+5-Aza	2.4	2.6	9.0	10.4	6.1	2.4	10.4
MV4-11R-pkc	58.5	34.5	48	46.5	46.9	34.5	58.5
MV4-11Rpkc+5-Aza	4.7	2.7	11.7	14.4	8.4	2.7	14.4
Low Meth Control	2.3	2.6	1.2	1.3	1.9	1.2	2.6
Med Meth Control	46.8	39.5	25.8	26.7	34.7	25.8	46.8
High Meth Control	96.7	85.3	49.3	50.4	70.4	49.3	96.7

It demonstrates methylation levels of 4CpG islands of PRG2 gene and shows low methylation in CpG islands of PRG2 in 5-Aza treated cells compared with other cells.

In conclusion, our findings support the hypothesis that TSG such as *SHP-1* and *PRG2* would lose their tumor suppressor function due to epigenetic silencing and their re-expression might enhance sensitivity responses to TKI. Thus, 5-Aza followed by PKC-412 or CEP-701 could provide suitable candidates as alternative option for the treatment of AML patients.

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