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Received 13 Mac 2018
Revised 17 May 2018.
Accepted 25 June 2018.
Published Online 30 June 2018

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Original Article

Apoptotic Induction in CCRF-CEM and HL-60 Human Leukemic Cell Lines by 5-Azacitidine and Trichostatin A

Abstract—The aims of the study were to investigate the anti-cancer effects of 5-Aza and TSA in two leukemic cell lines (CCRF-CEM and HL-60). Inhibition concentration of 5-Aza and TSA were measured using trypan blue exclusion assay. 5-Aza and TSA at IC₅₀ were treated to both CCRF-CEM and HL-60 cell lines for 4-6 days. To confirm the inhibition effects of these agents, Annexin-V stained cells were analyzed using flow cytometry to evaluate the apoptotic induction. The IC₅₀ values of CCRF-CEM were 2.01±0.1µM and 2.65±0.3µM for 5-Aza- and TSA-treated, respectively. Whereas, the IC₅₀ values of HL-60 were 1.98±0.2µM and 2.35±0.2µM for 5-Aza- and TSA-treated, respectively. To further substantiate the findings, the time-dependent exposure of both drugs was studied. CCRF-CEM cells were reduced to 49.4%±5.0, 49.4%±2.5 and 41.5%±6.6 by 5-Aza; 56.5%±7.0, 45.3%±4.2 and 40.2%±4.2 by TSA treatment at first, third and sixth day. HL-60 cells were reduced to 72.0%±4.5, 51.0%±1.5 and 40.6%±2.6 by 5-Aza at first, third and sixth day. Meanwhile, HL-60 cells reduced to 55.6%±4.5, 45.2%±4.0 and 36.3%±2.9 by TSA at first, second and fourth day. Both cell lines were significantly inhibited (p<0.05) compared to the untreated. Furthermore, flow cytometry demonstrated that 5-Aza and TSA significantly increased the cells population positive for Annexin-V in CCRF-CEM and HL-60 cell lines. In CCRF-CEM, the total apoptotic rates were 51.7%±9.7 and 49.4%±6.0 for 5-Aza- and TSA-treated, while, in HL-60, the apoptotic rates were 51.0%±3.9 and 49.7%±9.6 for 5-Aza- and TSA-treated, in a dose- and time-dependent manner, respectively. Epigenetic modification drugs, 5-Aza and TSA have anti-leukemic effects and induce apoptosis at micro-molar concentrations in CCRF-CEM and HL-60 leukemic cell lines. These results may provide a new insight into the use of 5-Aza and TSA in inhibiting the growth of leukemic cells and useful strategy in developing an epigenetic therapy.

Keywords—Epigenetic, 5-Aza, TSA, CCRF-CEM, HL-60

1 INTRODUCTION
Leukemia is a malignant and clonal proliferation of hematopoietic stem cells (HSCs) leading to bone marrow failure and tissue infiltration to most of the liver, spleen and lymph nodes [1]. HSCs lose control over their growth and differentiation but preserve their self-renewal capacity [2]. It is characterized as a heterogeneous disease; may arise at different stages of differentiation of the lymphoid and myeloid precursors [3]. Despite the advances in effective treatments for leukemia, however, the survival rate among those patients remain unsatisfactory.

Epigenetic modification of DNA methylation and histone modification resulted in transcriptional silencing and inactivation of tumor suppressor genes associated with all stages of cancer including initiation, progression, invasion and metastasis [4]. DNA methylation is a covalent addition of methyl group to carbon at position 5 of the cytosine within the CpG dinucleotides. Hypermethylation and hypomethylation occur in cancer that leads to genomic instability. Histone acetylation is regulated by histone acetyltransferases (HAT) and histone deacetylase (HDAC) which cause changes in chromatin structure by manipulating histone-DNA interaction. Deacetylated histones are associated with cell growth and hyperacetylated are associated with cell growth arrest, differentiation, and apoptosis [5].

However, these modifications can be reversible with potential synthetic drug targeting enzymes; 5-Azacitidine (5-Aza) and Trichostatin A (TSA), to re-express those silencing genes, a subject for epigenetic chemotherapy. 5-Aza is a

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demethylating agent, incorporates into both RNA and DNA to inhibit DNA methyltransferases (DNMTs), hypomethylation of DNA and induction of DNA damage [6], whereas, TSA inhibit deacetylation of both histone and non-histones cellular protein, inducing histone acetylation and chromatin relaxation result in genes transcription. 5-Aza has been approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [7, 8]. TSA is an antifungal antibiotic derived from Streptomyces hygroscopicus that inhibits mammalian histone deacetylase classes I and II and efficiently induces apoptosis in various cancer cell types including leukemia [9, 10].

These epigenetic modification drugs have the ability to reverse methylation status and histone acetylation which induce apoptosis [10-13]. Although epigenetic modulation effects have been intensively investigated in several cancers including leukemia, the effects of the epigenetic modification on T-acute lymphocytic leukemia (ALL) and acute promyelocytic leukemia (APML) has been ignored by others. Therefore, this study aimed to determine the effects of epigenetic modification on the proliferation and apoptosis of T-ALL and APML human leukemic cell lines after exposure to 5-Aza and TSA.

Material and method

The culture of cell lines

CCRF-CEM (human T-lymphocyte leukemia) cell line was donated by Integrated Centre For Research Animal Care and Use (ICRACU), International Islamic University Malaysia; HL-60 (human acute promyelocytic leukemia) cell line was donated by the Faculty of Health Science, Universiti Sultan Zainal Abidin; originally purchased from American Type Cell Culture (ATCC) (MD, USA). The cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, CA, USA) and 1% (v/v) penicillin-streptomycin (Invitrogen, CA, USA) at 37°C in a humidified atmosphere air with 5% CO₂.

Treatments for leukemic cell lines

CCRF-CEM and HL-60 leukemic cell lines were seeded in 6-well plates at a density of 1 x 10⁵ cells/well in 3 mL of medium and incubated for 24 h. The cell lines were treated with different concentrations of 5-Aza and TSA; 0.1 µM, 0.5 µM, 1 µM, 3 µM, 5 µM and 10 µM in triplicate. The concentration of 5-Aza and TSA that inhibited 50% of leukemic cell lines population at 48 and 72 h were determined by trypan blue exclusion assay. Cell lines were stained with 0.4% trypan blue solution in the ratio of 1:1. Viable cells were counted and recorded as the percentage of cell viability following treatment compared to the untreated (control). Results were plotted using Graph Pad Prism 6 (Graph Pad Software Inc., San Diego, CA, USA) to determine the half maximal inhibitory concentration (IC₅₀) of the epigenetic modification drugs.

Flow cytometry analysis of apoptosis

Annexin V-FITC/PI double staining was used for the measurement of apoptosis after 48 and 72 h of treatment at their IC₅₀ values. A total of 1 x 10⁵ cells were harvested by centrifugation at 1200 rpm for 5 minutes. The cells were stained with 5 µL fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI) for 15 minutes at room temperature (RT) in the dark. The apoptotic cells were detected by flow cytometry and analyzed using Flow BD Fac Canto II (Becton Dickinson, NJ, USA). Untreated cell lines served as a negative control.

Statistical analysis

Graph Pad Prism for Windows, version 6 (Graph Pad Software Inc., San Diego, CA, USA) was used to determine the half maximal inhibitory concentration (IC₅₀) values of the epigenetic modification drugs. Independent t-test was used to compare the drugs tested at concentration and time. Kruskal-Wallis test was used for Annexin V-FITC to compare untreated- and treated-groups and Mann-Whitney test to compare individual groups. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (SPSS Inc. version 22, Chicago, Illinois, USA). The results were considered to be significant when the p-value was < 0.05 for all analyses.

2 RESULTS

Inhibition effects of 5-Aza and TSA on CCRF-CEM and HL-60

The inhibitory effect of 5-Aza and TSA were determined based on the viability of human leukemic cell lines. Trypan blue exclusion assay showed that 5-Aza and TSA significantly increased the trypan blue-stained cells, indicating the loss of cell membrane integrity and cell death. Figure 1 indicated that 5-Aza and TSA treatment
inhibit CCRF-CEM and HL-60 cells growth in a dose-response curve and the 50% inhibition concentration (IC\textsubscript{50}) values were established for each drug (Table 1). Both drugs were sensitive to CCRF-CEM and HL-60; the percentage of cell viability reduced at a lower drug concentration of 0.1 up to 10 \(\mu\)M.

The IC\textsubscript{50} was determined by plotting the percentage of cell viability in the presence of increasing concentrations of 5-Aza and TSA (Figure 1). The percentage of CCRF-CEM cells viability were 97.8-85.2%, 87.7-77.1%, 70.8-50.5%, 39.8-32.5%, 29.8-19.1% and 11.6-8.3% upon treatment of 5-Aza with 0.1 \(\mu\)M, 0.5 \(\mu\)M, 1 \(\mu\)M, 3 \(\mu\)M, 5 \(\mu\)M and 10 \(\mu\)M, respectively. While, the percentage of CCRF-CEM cells viability were 97.4-45.2%, 73.1-63.3%, 56.4-50.0%, 47.4-43.2%, 29.1-22.7% and 12.7-10.3% upon treatment of TSA with 0.1 \(\mu\)M, 0.5 \(\mu\)M, 1 \(\mu\)M, 3 \(\mu\)M, 5 \(\mu\)M and 10 \(\mu\)M, respectively. After 72 h of incubation, the percentage of CCRF-CEM cells viability decreased when the concentration of both drugs increased.

The percentage of HL-60 cells viability after 72 h of incubation were 99.6-81.0%, 80.2-71.1%, 74.3-49.7%, 38.9-24.2%, 25.4-8.1% and 8.3-3.0% upon treatment of 5-Aza with 0.1 \(\mu\)M, 0.5 \(\mu\)M, 1 \(\mu\)M, 3 \(\mu\)M, 5 \(\mu\)M and 10 \(\mu\)M, respectively. Whereas, the percentage of HL-60 cells viability after 48 h of incubation were 98.4-89.9%, 85.5-79.5%, 73.6-52.5%, 52.2-27.3%, 36.2-12.2% and 14.5-7.2% upon treatment of TSA with 0.1 \(\mu\)M, 0.5 \(\mu\)M, 1 \(\mu\)M, 3 \(\mu\)M, 5 \(\mu\)M and 10 \(\mu\)M, respectively. 5-Aza and TSA reduced the percentage of HL-60 cells viability over the increased concentration of both drugs after 72 and 48 h of post treatment.

Figure 1 (A) showed that the CCRF-CEM cell line was more sensitive to 5-Aza treatment when compared to TSA with lower IC\textsubscript{50} (2.01±0.1 \(\mu\)M and 2.65±0.3 \(\mu\)M, respectively). Furthermore, at concentration > 1 \(\mu\)M, 5-Aza consistently reduced cell viability by 10-30%, in contrast, TSA reduced cell viability below 20%. The maximum percentage of cell viability reduced differed between 5-Aza and TSA in 9 days, CCRF-CEM cell line in 9 days. CCRF-CEM cell line proliferation was significantly inhibited by 5-Aza and TSA (\(p<0.05\)) compared to the untreated cell line. However, there was no significant difference between the response of the cell lines towards treatments and the time of exposure.

Meanwhile, the HL-60 cell line was treated at IC\textsubscript{50} of 5-Aza and TSA in 9 and 6 days, respectively. HL-60 viable cells count was reduced to 74.4%, 50.1% and 42.2% by 5-Aza at first, third and sixth day (Figure 2B, i). The results also showed that HL-60 cell line was reduced to 73.3%, 50.0% and 32.1% by TSA at first, second and fourth day (Figure 2B, ii). In apposition to day 9 and 6 of 5-Aza and TSA treatments, the percentage of HL-60 viable cells slightly reduced to 38.0% and 19.7% by 5-Aza and TSA. The HL-60 cell line proliferation was significantly inhibited by 5-Aza and TSA (\(p<0.05\)) compared to the untreated cell line. However, there was no significant difference between the response of the cell towards treatment and the time of exposure time.

**Apoptosis induction of 5-Aza and TSA**

To confirm the inhibition effect of 5-Aza and TSA, flow cytometry analysis of apoptosis was performed. The morphological changes of CCRF-CEM and HL-60 cells were substantially observed; cell membrane blebbing, apoptotic bodies, nuclear fragmentation, and irregular shape under the fluorescent microscope (Figure 3). All were the typical changes of apoptosis induction due to the treatments.

Exposure of CCRF-CEM and HL-60 cells to 5-Aza and TSA significantly increased the cells population positive for Annexin V (Figure 4). The percentages of cells inhibition associated with specific apoptosis induction were determined (Figure 5). In CCRF-CEM cell line, the total apoptotic rates were 51.7% ± 9.7 and 49.4% ± 6.0 by 5-Aza and TSA in a dose- and time-dependent manner, respectively. Whereas, in the HL-60 cell line, the apoptotic rates were 51.0% ±
3.9 and 49.7% ± 9.6 by 5-Aza and TSA in a dose- and time-dependent manner, respectively. The results suggested that 5-Aza treatment on CCRF-CEM was more responsive in inducing apoptosis than TSA. On the other hand, TSA resulted in higher apoptosis in HL-60 whereby almost half of HL-60 cells population died within 48 h.

**Table 1:** The IC<sub>50</sub> values of 5-Aza and TSA from the dose-response curve for leukemic cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time (h)</th>
<th>5-Aza IC&lt;sub&gt;50 ± SEM&lt;/sub&gt; (µM)</th>
<th>TSA IC&lt;sub&gt;50 ± SEM&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>72</td>
<td>2.01±0.1</td>
<td>2.65±0.3</td>
</tr>
<tr>
<td>HL-60</td>
<td>48</td>
<td>-</td>
<td>2.35±0.2</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.98±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Graphpad Prism software.
SEM = standard error mean.

**Figure 1:** Effects 5-Aza and TSA in human leukemic cell lines. (A) Percentage of CCRF-CEM viability was determined after 72h of treatment with 5-Aza and TSA. (B) Percentage of HL-60 viability was determined after 72 and 48h of treatment with 5-Aza and TSA, respectively. Each point is the mean value of three replicate experiments. P-values are for individual treatment groups compared to untreated (p<0.05). Data were plotted using Graph Pad Prism Version 6.

**Figure 2:** Time-dependent effect of 5-Aza and TSA on CCRF-CEM and HL-60. (A) 5-Aza and TSA on CCRF-CEM (B) Effect 5-Aza on HL-60. (C) Effect TSA on HL-60. Results represent the median. P-values are for individual treatment groups compared to control (p<0.05).
**DISCUSSION**

Epigenetic therapy becomes more interesting field due to its very prevalent and stable changes in DNA methylation while histone deacetylase activity has been shown in early clinical trials in AML and ALL patients [14]. In this study, the potential effects of epigenetic modification drugs; DNA methylation inhibitor (DNMTi) and histone deacetylase inhibitor (HDACi) were investigated as a promising strategy for leukemia treatment.

![Figure 5: FACS analysis of apoptosis in treated cell lines. (A) CCRF-CEM and (B) HL-60 cells. Results represent the medians ± interquartile range. Kruskal Wallis test, there was a significant difference of medians apoptosis between four treatment groups. Based on Mann Whitney test on an individual pair, on only 5-Aza and untreated; 5-Aza and CEP; TSA and untreated; TSA and CEP; untreated and CEP were significantly different at p<0.05 by Bonferroni’s procedures. One-way ANOVA test, there was no significant difference between CCRF-CEM and HL-60 cells. CEP = Cep-701 (Lestaurtinib), a tyrosine kinase inhibitor, as a positive control.](image-url)

![Figure 4: Induction of apoptosis in CCRF-CEM and HL-60 cells by Cep-701 as a positive control. (A) CCRF-CEM cells treated with 5-Aza and TSA, 72 h post treatment. (B) HL-60 cells treated with 5-Aza and TSA, 72 and 48 h post treatment, respectively.](image-url)

![Figure 3: The morphology characteristics of untreated, and treated CCRF-CEM and HL-60. The typical features of apoptosis; membrane blebbing, apoptotic bodies, nuclear fragmentation and cytoplasmic shrinkage.](image-url)
Apoptosis study is a hallmark of cancer research as it is important to outline the biomarkers for morphological and molecular changes. Thus, we demonstrated that 5-Aza and TSA were effective as DNMT and HDAC inhibitors, promoting cell death and inducing apoptosis of human leukemia cell lines.

Studies on differentiation therapy of T-ALL induced by anti-cancer compounds are still limited. Differentiation therapy is defined as in vitro studies that show a variety of agents stimulate differentiation of the cell lines [15]. This was due to ALL has been classified as a disease of differentiated lymphocytes. However, ALL progenitor could undergo terminal differentiation; because most of ALL cases arise from transformed immature hematopoietic stem cells with self-renewal and differentiation capacity [16]. Therefore, we decided to investigate the inhibition effect and apoptosis induction in CCRF-CEM. Interestingly the result showed that 5-Aza and TSA significantly increased the percentage of cell inhibition and apoptosis induction. It is suggested that CCRF-CEM cells were susceptible to both drugs. Our finding was consistent with a previously published data suggested that CCRF-CEM cell lines have the capacity to respond to the epigenetic modification drugs [13].

The IC_{50} values showed that CCRF-CEM cell line was more sensitive to 5-Aza than TSA. Our findings suggested that 5-Aza treatment at low concentration may affect DNA methylation in CCRF-CEM. Although we cannot rule out the effects of 5-Aza treatment on DNA methylation process, a number of reports suggested that 5-Aza was highly sensitive to inhibit human leukemia cells at a concentration with less than 5 µM [8, 11, 17]. Whereas in HL-60, TSA was more responsive compared to 5-Aza as it took a shorter time range. This result suggested HL-60 cells with low autophagy had triggered TSA to induce apoptosis by repressing autophagy and lead to accumulation of mitochondria, production of reactive oxygen species and DNA-damage [18]. Our results were also consistent with previous literature that IC_{50} of TSA was moderate (0.5-5 µM) for anti-leukemia activities based on reduced cell viability [19, 20]. 5-Aza and TSA had specifically inhibited the proliferation of CCRF-CEM and HL-60 cell lines, thus, making them suitable agents targeting for cancer cells.

Collectively, compared to CCRF-CEM, HL-60 was more sensitive to the inhibitory effects of both 5-Aza and TSA. As shown in our results, the half-lives of 5-Aza and TSA in HL-60 were in short duration of incubation, i.e within 48-72 h. These findings were consistent with previous data reported that 5-Aza and TSA had their maximal reduced cell viability observed in 48 h and 72 h [11, 20, 21]. Furthermore, the sensitivity was probably due to subtypes of leukemia; different phenotypes, diverse chromosomal abnormalities and epigenetic changes patterns [14, 20]. HL-60, derived from acute promyelocytic leukemia (APML) by PML/RARα fusion protein, tends to be less aggressive and highly sensitive to respond to epigenetic modification drugs [5, 14].

We further exposed CCRF-CEM and HL-60 to 5-Aza and TSA for 6 and 9 days. However, the maximal response was only seen on the third day except for the second day for HL-60 treated with TSA. 5-Aza and TSA were not sufficient in inhibiting CCRF-CEM and HL-60 cells viability during the extended time as they were only exposed once (at day zero). Thus, to exert the therapeutic effect, it is suggested to regularly treat the cell lines e.g. for every second and third day. This was consistent with the finding of Yang, Hoshino [14] and Hollenbach, Nguyen [11] that prolonged used (daily exposure of tested drug for 10 days) was shown to be a well-tolerated and had a significant effect.

5-Aza and TSA induced apoptosis in CCRF-CEM and HL-60 as shown by flow cytometry analysis. We found that the treated cell lines exhibited the typical features of apoptosis. The apoptotic cells disassembly in treated CCRF-CEM and HL-60 suggested that the flip-flop of phosphatidylserine (PS) to the outer layer of the cell membrane was promoted by 5-Aza and TSA. These results suggested that both epigenetic modification drugs exerted their anti-cancer effect by epigenetic-mediated programmed cell death. This was supported by several studies demonstrated apoptosis induction in leukemia cell lines by 5-Aza and TSA [6-8, 13, 22].

The percentage of apoptosis including early and late apoptotic cells increased significantly (p<0.05) in a dose- and time-dependent manner. However, between different groups of treatment, 5-Aza and TSA were not significant (Figure 5). Even though we could not show the fine-tuning of reversible action of both epigenetic modification agents that target leukemia cells, however, this result suggested that 5-Aza and TSA were effective against CCRF-CEM and HL-60. Both drugs had been reported to have their reversible action on leukemia cells and consequently lead to cell death [8, 18, 22, 23]. Furthermore, the flow cytometric analysis
demonstrated cell inhibition was due to the mechanism of DNMTi- and HDACi-induce apoptosis that selectively target leukemia cells.

CONCLUSION
In conclusion, this study demonstrated that 5-Aza and TSA have an anti-leukemic effect on T-ALL derived (CCRF-CEM) and APML derived (HL-60) cell lines. We have shown a consistent inhibition effect and apoptosis induction in both cell lines at acceptable IC50. Both drugs induce apoptosis towards human leukemic cells lines in a dose- and time-dependent manner. Thus, 5-Aza and TSA are suggested as potential epigenetic therapy for leukemia treatment in the future. Further study of in vitro, in vivo and clinical trials, are needed to verify whether 5-Aza and TSA are the feasible alternatives in treating leukemia.

CONFLICTS OF INTEREST
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

ACKNOWLEDGEMENT
The authors acknowledge the Ministry of Higher Education (MOHE) for financial support under the Fundamental Research Grant Scheme (FRGS) 203/PPSP/6171164 and PPSP Incentive Postgraduate Studies Development Fund (TIPPS 2017), USM. Appreciation goes to Craniofacial Laboratory, School of Dental Sciences, Molecular Laboratory, Department of Hematology and Central Research Laboratory (CRL), School of Medical Sciences, USM for the facilities.

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