Arsenic Trioxide and Thalidomide on Expression of Vascular Endothelial Growth Factor

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Abstract

Acute myeloid leukemia (AML) is a blood disorder characterized by uncontrolled proliferation of myeloid progenitors and decrease in the apoptosis rate. The vascular endothelial growth factor (VEGF) promotes blood vessel regeneration which might play important roles in development and progression of neoplasia. Our previous studies focused on cytotoxicity and anticancer effects of arsenic trioxide (ATO) and thalidomide (THAL) as an anti-VEGF compound in the AML cell model. ATO also affects regulatory genes involved in cell proliferation and apoptosis. The aim of present study was to examine the effects of ATO and THAL alone and in combination on U937 and KG-1 cells, with attention to mRNA expression for VEGF isoforms. Growth inhibitory effects was assessed by MTT assay and apoptosis induction was determined by Annexin/PI staining. mRNA expression levels were evaluated by real-time PCR. Our data indicated that ATO (1.618μM and 1μM in KG-1 and U937 cell lines respectively), THAL (80μM and 60μM) and their combination inhibited proliferation and induced apoptosis in our cell lines. mRNA expression of VEGF (A, B) decreased while C and D isoforms did not show any significant changes. Taken together, according to the obtained results, the VEGF autocrine loop could be a target as a therapeutic strategy for cases of AML.

Keywords: Arsenic trioxide- Thalidomide- Vascular Endothelial Growth Factor (VEGF)- acute myeloid leukemia

Introduction

Acute myeloid leukemia (AML) is the heterogeneous malignant which is characterized by the uncontrolled proliferation of hematopoietic stem cells and myeloid progenitors and a decrease in the apoptosis rate. The vascular endothelial growth factor (VEGF) promotes blood vessel regeneration which might play important roles in development and progression of neoplasia. Our previous studies focused on cytotoxicity and anticancer effects of arsenic trioxide (ATO) and thalidomide (THAL) as an anti-VEGF compound in the AML cell model. ATO also affects regulatory genes involved in cell proliferation and apoptosis. The aim of present study was to examine the effects of ATO and THAL alone and in combination on U937 and KG-1 cells, with attention to mRNA expression for VEGF isoforms. Growth inhibitory effects was assessed by MTT assay and apoptosis induction was determined by Annexin/PI staining. mRNA expression levels were evaluated by real-time PCR. Our data indicated that ATO (1.618μM and 1μM in KG-1 and U937 cell lines respectively), THAL (80μM and 60μM) and their combination inhibited proliferation and induced apoptosis in our cell lines. mRNA expression of VEGF (A, B) decreased while C and D isoforms did not show any significant changes. Taken together, according to the obtained results, the VEGF autocrine loop could be a target as a therapeutic strategy for cases of AML.

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Bcl-2 family members and inhibits the NF-κB activation (Miller et al., 2002). In addition, ATO prevents the angiogenesis by inhibiting the cell growth (Lew et al., 1999). ATO causes down-regulation of VEGF expression and increases the apoptosis (Roboz et al., 2000) (Figure 1). THAL has anti-angiogenesis effects on tumour growth and progression (Woodyatt, 1962; Salemi et al., 2017). This agent inhibits the angiogenesis of basic fibroblast growth factor (β-FGF) in rabbit and VEGF in murine (D’Amato et al., 1994; Kenyon et al., 1997). Due to the anti-angiogenesis property of THAL, it has been used for treatment of various solid tumours, multiple myeloma, and other hematologic malignancies (Figg et al., 1997; Eisen et al., 1998; Long et al., 1998; Marx et al., 1999; Drake et al., 2003). (Figure 2) Hence, the aim of this study was to evaluate the combination effects of ATO and THAL as a new strategy with anti-VEGF properties and induction of apoptosis in leukemic cell lines.

Materials and Methods

Reagents
THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water. THAL was purchased from Santa Cruz Company (Santa Cruz, Dallas, Texas); and ATO was obtained from Sina Darou Company (Tehran, Iran). 5-diphenyltetrazolium bromide (MTT) dye, Annexin V-FITC apoptosis detection kit, Dimethyl Sulfoxide (DMSO) and DEPC treated water. ATO was dissolved in distilled water.
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Flow cytometry was performed to assess the effect of cited compound on apoptosis induction. Annexin-V/PI staining indicated that ATO and THAL induced the apoptosis. According to the obtained data, we observed significant increase in percentage of early apoptosis significantly reduced the proliferation of cited leukemic cells. ATO and THAL reduce proliferation of KG-1 and U937 in a dose-dependent manner (Figure 3: A-C and Figure 4: A-C).

Apoptosis

Table 1. Primer Used for qRT-PCR

| Gene   | Forward Primer(5'-3')                  | Reverse Primer(5'-3')                  | Size(bp) | Ref                  |
|--------|----------------------------------------|----------------------------------------|----------|----------------------|
| GAPDH  | TGAACGGGAAGCTCACCTG                  | TCCACCAACTGTGATGCTG                   | 19       | (Kong et al., 2014b) |
| HPRT   | GCTATAATATTTTCTGCACCTGTG               | AATTACTTTATATGGCCTCCTGTAGCTG          | 26       | (Gusenbauer et al., 2015) |
| VEGF-A | AGGCCTATCCTACGGAATGT                   | AGGGMTCGATTGGGATGGCA                   | 21       | (Kong et al., 2014a) |
| VEGF-B | GAGATGTCCCTGAGAACAAAAACACAA           | GAGGAGATGCCGGCTGTATGTCAG               | 22       | (Yang et al., 2009)  |
| VEGF-C | GAGGAGCAGTTACGCTCAGCTG                | TCTTTCCCTTAGCTGACACTGTG               | 21       | (Awad et al., 2015)  |
| VEGF-D | GTATGGACTTCGTCAGCAT                   | AGGCTCTCTTCAATGCAACAG                 | 21       | Takeshi Terabayashi et al., 2015 |

Figure 1. Targeting of Signaling Pathways by ATO in AML Cells. ATO treatment of leukemic cells results in inhibition of the PI3K/Akt pathway; and pharmacologic targeting of this pathway enhances the antileukemic effects of ATO. The potential involvement of other MAPK pathways, such as the p38 MAPK and MEK/ERK pathways, which play important roles in the control of growth and survival of other types of leukemic cells. ATO could suppress angiogenesis factor indirectly by suppress PI3K/AKT pathway.

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Apoptosis
apoptotic cells and minimal percentage of necrotic cells was observed when compared with the control. In addition, significant increase of apoptotic cells (61% in KG-1 and 88% in U937) were seen in combination of ATO and THAL.

**Cell Cycle analysis**

DNA content of KG-1 and U937 cells assessed after treatment with cited compounds and their combination by PI staining. According to the cell cycle analysis we observed G1 area increased in KG-1 and U937 cells when treated with ATO and THAL and their combination. The percentages of cells at G2 phase was simultaneously reduced in all treated cells. Therefore, it seems that combination of ATO and THAL induce sub G1/G1 arrest (5.71% to 16.32% for KG-1 cell and 5.05% to 36.87% for U937) in both cell lines (Figure 7A and 7B).

**Real-Time PCR assay**

KG-1 and U937 cells were treated with certain concentrations of ATO and THAL for 48h, and they were then examined for expression of VEGFA, VEGFB, VEGFC, VEGFD by the Real-Time PCR. According to these results, the expression level of VEGFA and VEGFB was significantly lower in treated cells than the untreated cells with maximum effect at the concentrations of 80 µM and 60µM of THAL on KG-1 and U937 cell lines and 1.618µM and 1 µM of ATO on KG-1 and U937 respectively (Figures 8A and 8B).
Figure 5. Flow Cytometry of KG-1 Cells Treated with ATO (1.618 µM) and THAL (80µM) and Their Combination. The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

Figure 6. Flow Cytometry of U937 Cells Treated with ATO (1µM) and THAL (60µM) and Their Combination. The lower left quadrant shows live cells; the lower right, early apoptotic cells; the upper right, late apoptotic cells and the upper left quadrant shows necrotic cells. Statistical significance were defined at *P<0.05, **P<0.01 and ***P<0.001 compared to corresponding control.

Figure 7. Cell Cycle Flow Cytometry Analysis of Leukemia Cells. 48h exposure to different concentrations of ATO and THAL reduces the number of cells at G2 phase and increases the amount of cells at G1 phase of the cell cycle in KG-1, and U937 cells and was observed significant accumulation of cells in theG0 /G1 phase.. Statistical significance were defined at * p <0.05, ** p <0.01 and *** p <0.001 compared to corresponding control.
AML is a type of cancer which is characterized by the infiltration of blasts to the bone marrow, blood, and other tissues (Döhner et al., 2015; Mohammadi et al., 2017c; Zahed Panah et al., 2017). VEGF is one of the critical regulators of angiogenesis and it affects the proliferation of both solid tumours and haematological malignancies (Rodriguez-Ariza et al., 2011). In the present study, we investigated the effects of ATO and THAL on expression of VEGFA, VEGFB, VEGFC, and VEGFD in the leukemic cell lines. AML blast cells cause aberrant expression of VEGF ligands and receptors. The higher VEGF ligand expression is significantly associated with a poor outcome in AML patients. AML blasts enhance the autocrine VEGF signalling in order to increase the proliferation and provide a survival advantage. From therapeutic aspects, VEGF is a well-known target for treatment of AML. VEGF-targeted therapy in the AML patients could inhibit the autocrine VEGF signalling in AML cells as well as the aberrant vessel formation by the vascular endothelial cells (Kampen et al., 2013).

ATO prevents the cell proliferation and induces apoptosis in some cancer cells including the solid tumors and acute promyelocytic leukemia. ATO affects different signaling pathways. Study on the mechanisms of ATO may be useful in designing more effective cancer therapies (Siu et al., 2002; Yu et al., 2007; Morales et al., 2008; Liu et al., 2012).

THAL is an immunomodulatory agent, it can be used as a potential compound for treatment of malignant and immunological disorders. This agent inhibits the angiogenesis by blocking basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Kruse et al., 1998), modulates various cytokines (Corral et al., 1999), enhances cell-mediated immunity through direct co-stimulated T-cells (Haslett et al., 1998), and alters adhesion molecule expression (Geitz et al., 1996).

The present research investigated the cytotoxic effects of ATO and THAL and also the induction of apoptosis in both U937 and KG-1 cell lines. Results of the flow cytometry indicated that these selected doses could induce a significant percentage of apoptosis. Besides, the previous studies indicated the concentration range of 0.5-2 µM (Zhang et al., 1998; Song et al., 2012; Noguera et al., 2017) for induction apoptosis by ATO; hence, the selected single doses of ATO were 1.618µM and 1µM for KG-1 and U937 respectively.

Cell cycle regulation is typically damaged in leukemic cells. ATO induces the cell cycle arrest at G1 in leukemic cells (Noguera et al., 2017). THAL also induces the apoptosis and cell cycle arrest at G1 phase in leukemic cells (Steins et al., 2002). According to the above-mentioned studies, ATO and THAL arrested cell cycle at Sub G1/G1 in both cell lines. According to the previous researches, ATO induces the apoptosis in leukemic cells and blood vessel endothelial cells by a time/dose-dependent manner via inhibition of the VEGFA production (Ge et al., 2015). THAL and other immunomodulatory agents (e.g. lenalidomide) were studied in multiple myeloma which showed a significant decreased in expression of pro-angiogenic factor VEGF and interleukin-6 (IL-6) (Gupta et al., 2001). Real-time data analysis indicated that ATO and THAL down-regulated gene expression of VEGFA, VEGFB, VEGFD and up-regulated the VEGFC in both cell lines. In consistent with our results, THAL induced anti-angiogenesis by at lower levels of FGF-2 and VEGF (Raza et al., 2008), Roboz et al., (2000) found that ATO induced apoptosis in leukemia by inhibiting VEGF. Yang et al., (2014) on the lung cancer model found that the ATO inhibited factors such as VEGF-A, VEGFR-2, HIF-1α and Notch-1, angiogenesis pathway (Yang et al., 2014).

In conclusion, to sum up, THAL as a VEGF inhibitor in combination with ATO has a synergistic impact on the inhibition of cell proliferation and promotion of apoptosis in AML cell lines. ATO enhances the anti-leukemic activity of THAL in both U937 and KG-1 cell populations when it is simultaneously used in combination.

Acknowledgments

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