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Trisenox induces cytotoxicity through phosphorylation of MAPK molecules in acute leukemia cells

Sanjay Kumar¹, Ibrahim O. Farah², and Paul B. Tchounwou^{1,*}

¹Cellomics and Toxicogenomics Research Laboratory, NIH/NIMHD-RCMI Center for Environmental Health, College of Science, Engineering and Technology,

²Department of Biology, Jackson State University, 1400 J.R Lynch Street, Box18750, Jackson, Mississippi, MS39217, USA.

Abstract

Trisenox (TX) has been used successfully for the treatment of acute promyelocytic leukemia (APL) patients. TX –induced cytotoxicity in APL cells remains poorly understood. In this research, we investigated the molecular mechanism of TX cytotoxicity using APL cell lines. We assessed TX toxicity by quantitatively measuring lactate dehydrogenase (LDH) levels. Inhibition of cell cycle progression was assessed by confocal microscopy of Ki67 expression. Apoptosis was evaluated by western blot analysis of apoptotic proteins expression, immunocytochemistry, and confocal imaging of annexin V and propidium iodide. MAPK signaling cascade was analyzed by western blotting and inhibitor (based experiments with APL cells. We found that TX (induced cytotoxicity inhibited APL cell cycle progression. TX also induced significant ($p<0.05$) changes in the expression levels of apoptotic molecules, and activated the phosphorylation of MAPK signaling pathways in APL cells. Understanding the mechanism of TX cytotoxicity would be help in the design of new APL drugs.

Keywords

Trisenox; cytotoxicity; APL cell line; apoptosis; MAPK signaling

1. INTRODUCTION

Acute promyelocytic leukemia (APL) is a blood cancer that develops inside the bone marrow cells as a consequence of chromosomal translocation between the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor alpha (RAR α) gene on chromosome 17 [1–3]. It represents approximately 5(10% of all cases of acute myeloid leukemia (AML) [4]. APL strikes around 1500 patients of all age groups annually in United States and accounts for nearly 1.2% of cancer deaths [5–7]. Trisenox (TX) has been used

*Correspondence Paul B. Tchounwou, paul.b.tchounwou@jsums.edu, Tel. 601-979-0777; Fax. 601-979-0570.

Authors' contributions

SK and PBT conceived, designed and implemented the study, and drafted the manuscript. IOF provided support in research implementation. All authors read and approved the final draft of the manuscript.

Competing interests

The authors declare that they have no competing interests.

successfully for the treatment of both relapsed and refractory APL patients, either alone or combination with all trans retinoic acid (ATRA) to achieve complete remission and maximum survival rate [8–13]. Even without ATRA combination, TX has been used successfully in APL treatment and has resulted in complete remission of patients with maximum survival rate [8, 14]. TX-treated APL patients also achieved a high rate of 5 (years disease free survival (DFS) and an overall survival (OS) in Phase II clinical trials [15].

Several reports have pointed out that lower concentrations of TX induce cell differentiation, whereas higher concentrations inhibit cell proliferation, and activates apoptosis in APL cells [16, 17]. Earlier *in vitro* studies have reported that TX produces a dual effect in APL cells; by inducing a partial cell differentiation at lower concentrations (0.1–0.5 μ Mmol/l), and apoptosis at higher concentrations (0.5–2.0 μ Mmol/l) as a result of rapid degradation of PML(RAR α) proteins and activation of caspases [16–19]. *In vivo*, TX has been reported to eradicate leukemia– initiating cells in a murine model of APL by activating promyelocytic leukemia(tumor protein 53 axis, and inducing an effective remission leading to a potential cure of APL [16, 20]. Although the detailed molecular mechanisms of the anti(leukemic potency of TX are not well known, it has been reported to inhibit proliferation of human myeloma cells through cell cycle arrest and apoptosis [21]. TX has also been reported to induce cell cycle arrest and apoptosis in lymphoid neoplasms [22] and NB4 cells [23]. It has also been reported to induce oxidative stress, DNA damage, and changes in the expression and translocation of apoptotic proteins in hepatocellular carcinoma cells, HL-60, NB4, leukemia and myeloma cells [1–7, 24–27].

Cytotoxicity is a complex process mediated through DNA-adduct, oxidative stress, transcriptional factors and stress signaling leading to cell death [3]. TX combination with cisplatin enhances cytotoxicity in head and neck squamous cell carcinoma cells [28] and small cell lung cancer cells [29]. This drug combination has also helped to overcome drug resistance and enhance cytotoxicity in APL cells. Few reports have suggested that TX induces cytotoxicity via alteration of redox homeostasis and mitochondrial integrity in lung cancer cells [30] and breast cancer cells [31].

Research has also pointed out that TX activates MAPK signaling pathway in different cancer cell lines. It has been found to induce apoptosis in acute myeloid leukemia (M2)(derived cell line, NKM-1 by activating c-jun (terminal kinase (JNK/SAP) pathway [32], human cervical cancer cells by activating JNK and p38 MAPK signaling pathways [33]. However, TX effect on MAPK signaling has not been fully elucidated. Therefore, we are reporting here that MAPK signaling cascade is involved in TX(induced cytotoxicity, cell cycle inhibition, and programmed cell death in APL cells. This research finding provides a scientific basis for identifying novel molecular targets of TX in APL chemotherapy.

2. MATERIALS AND METHODS

2.1 Cell Line and Culture

APL cell lines (NB4, HL-60, and KG1a cells) were used in this study. They were purchased from the American Type Culture Collection (Manassas, VA), and maintained at 37°C in an atmosphere of 5% CO₂ and 95% air according to standard procedures. They were cultured

in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum (FBS), and 1% penicillin(streptomycin solution, with a cell density of 2×10^5 viable cells/ml. Approximately 5×10^7 cells were seeded for each concentration of TX and incubated for 24 hours at 37 ° C inside a CO₂ incubator.

2.2 Chemicals and Reagents

TX, and apoptosis kit including Annexin V and propidium iodide [Cat # V13241] were purchased from Fischer Scientific (Pittsburgh, PA). Cytotoxicity assay kit [cat # G1780] was obtained from Promega (Madison, WI). Anti(Cytochrome C, anti-Bax, anti-p-P38MAPK, anti(p-Erk, anti- p-JNK and anti(Bcl2 were purchased by Cell Signaling Technology (Danvers, MA). Ki67, Hoechst 33342, Alexa fluor 568 and Alexa fluor 568 were purchased from Life Technologies (Grand Island/NY), SB203580 [Cat # S8307] and SP600125 [Cat # S5567] were obtained from Sigma(Aldrich (St. Louis, MO).

2.3 Cytotoxicity Assay

APL cells were grown in absence or presence to different concentrations (2, 4, 6, and 8 mg/ml) of TX for 24 hrs inside a CO₂ incubator, maintaining a standard temperature (37 ° C) and a 5% CO₂ level. After incubation, the cells were centrifuged and the supernatant was collected from all samples. LDH release in control and TX(treated APL cells supernatant was measured by following the Promega non(radioactive cytotoxicity assay technical bulletin protocol (Cat # G1780) and as described earlier [3]. Experimental data were presented as % of LDH release in TX(treated APL cells at different concentrations (2,4, 6, and 8mg/ml) compared to total LDH released in control cells.

2.4 Immunocytochemistry and confocal imaging

APL cells (1×10^5) were cultured in presence or absence of different concentrations (2, 4, 6, and 8mg/ml) of TX for 24hrs. After incubation, cells were attached on poly(L(lysine coated slide, and ki67 staining was performed as described earlier [3]. In brief, incubated cells were fixed by using 3% paraformaldehyde and permeablized with 0.2% NP-40 containing 0.5% glycine. Fixed and permeabilized cells were blocked in 4% BSA and incubated overnight in Ki-67 antibody (dilution, 1:100) (cat# 33-4711) from Life Technology company at 4 °C. After incubation, cells were washed with PBS three times and tagged with secondary antibody (anti-mouse) for one hour at room temperature followed by Hoechst 33342(dilution, 1:2000) staining for 7 min. Slides were washed with PBS and mounted coverslip using prolong gold an tifade reagent. After drying, slides were imaged by confocal microscopy (Olympus company, Center valley, PA).

2.5 Apoptosis assay

APL cells (1×10^6) were treated with different concentrations of TX for 24hrs. After treatment, both early and late apoptosis were assayed using FITC an nexin V apoptosis detection kit I (BD biosciences; Cat. No. 556547). In brief, both control and TX treated cells were washed with ice cold phosphate buffer saline (PBS) and incubated 15 min in mixture of an nexin V and propidium iodide (PI) at room temperature. After incubation, cells were

washed with 1X binding buffer and apoptosis analyzed by cello meter vision CBA (Lawrence, MA) / confocal microscopy (Fluoview 10i, Olympus; Center Valley, PA).

2.6 Western blotting analysis

APL cells were treated with different concentrations of TX for 24 hours at 37 ° C. After treatment, cells were washed twice with cold phosphate buffered saline (PBS) and lysed in RIPA buffer containing (1% Nonidet P(40, 0.5% sodium deoxycholate, 0.1% SDS, 100Mg/ml phenylmethylsulfonyl fluoride, 100 Mg/ml aprotinin, 1 Mg/ml leupeptin, and 1 mm sodium orthovanadate) on ice 20 minutes. It was centrifuged at 14000 rpm for 12 minutes and supernatant collected in fresh micro centrifuge tubes. We have used supernatant as cell extracts. Cell extracts were prepared, protein estimated, and 40mg of protein from control or treated cells were loaded per lane on a 10% SDS(PAGE gel, transferred into nitrocellulose membrane, and analyzed by western blotting using specific antibodies as described previously [1,3]. The band intensities were quantified using Image J (National Institutes of Health).

2.7 Pharmacological inhibitor studies of MAPK pathways

HL-60 cells were incubated with SB203580 (specific inhibitor of P38 MAPK) and SP600125 (specific inhibitor of JNK) for 30 min followed by TX treatment. Cell lysates were prepared using RIPA buffer along with protease inhibitors and phosphates inhibitor (sodium petaphosphate). MAPK molecules phosphorylation inhibition studies were performed using MAPK signaling molecule inhibitors and TX-treated cells lysates. They were then analyzed by western blotting using phopho-specific P38 MAPK (cat# 9211) and JNK (cat # 9251) antibodies.

2.8 Statistical analysis

Experiments were performed in triplicates. Data were presented as means $\pm$ SDs. Where appropriate, one-way ANOVA or student paired *t*-test was performed using SAS Software available in the Biostatistics Core Laboratory at Jackson State University. P-values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Trisenox causes cytotoxicity in APL cells

In the present study, we investigated the cytotoxic effect of Trisenox (TX) in APL cell lines (HL-60, KG(1a and NB4 cells) after 24 hours treatment with various concentrations (2, 4, 6, and 8 mg/ml) of TX in triplicate. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme released from damaged cells. It interact with tetrazolium salt (violet color) and changes into red formazan. Generation of a red formazan product is proportional to the amount of LDH released and therefore the number of damage cells. The amount of LDH released in the medium was determined by measuring the absorbance at 490nm. Our results showed that TX induces cytotoxicity significantly ($p<0.01$) in HL-60, KG-1a, and NB4 cells [Fig. 1A-C].

3.2 Trisenox inhibits cell cycle progression

TX(induced cytotoxicity modulated cell cycle progression in APL cells. We study TX effects on cell cycle progression in growing cells, presence or absence of different concentrations (2,4, 6, and 8 mg/ml) of TX in triplicate for 24hours. After treatment, we made confocal slides of all cells and performed imaging to check the expression of Ki67 protein, a cell cycle progression marker [34]. Our findings showed that TX inhibits cell cycle progression by downregulating the expression level of Ki67 in KG1a cells in a concentration(dependent manner [Fig. 2i-v].

3.3 Trisenox induces apoptosis

We analyzed TX(induced apoptosis in APL cells after treatment with different concentrations for 24 hours at 37 ° C. After treatment, cells were washed with cold PBS and further incubated with mixture of FITC tag annexinV, an early apoptotic marker [35], and propidium iodide (PI), a late apoptosis marker [36] at room temperature. We performed both western blotting and confocal imaging to assess the expression levels of apoptotic proteins (Bax, Bcl(2, and cytochrome C), caspase 3, annexin V, and PI. Our results showed that TX induces apoptosis in KG1a cells by increasing the expression levels of Bax, cytochrome C, caspase 3, annexin V, and PI, in a concentration-dependent manner [Fig. 3A-B(i-v)]. It also decreased the expression level of anti-apoptotic protein, Bcl(2 in KG1a cells in a concentration(dependent manner [Fig.3A].

3.4 Trisenox modulates MAP kinase signaling pathway

Recent reports have shown that arsenic trioxide induces apoptosis by stimulating MAPK signaling pathway in many cancer cells including leukemia cancer cells [37–39]. We have tested oxidative stress and modulation of stress(related signaling involvement in apoptosis in APL cells. All three molecules (JNK, Erk1/2 and P38MAPK) of MAPK signaling pathway were highly modulated in TX(treated HL-60 cells. However, phosphorylation of Erk1/2 and P38MAPK molecules were activated, whereas JNK phosphorylation was down regulated in KG1a cells. TX-induced phosphorylation of these molecules was significantly increased ($p<0.01$) in a concentration(dependent manner [Fig. 4A-B]. We have also performed specific pharmacological inhibitor studies of p38 MAPK signaling pathway using SB203580 (a specific inhibitor of P38 MAPK) [40] and SP600125 (a specific inhibitor of JNK) [41] to confirm that MAPK pathway is involved in TX –induced apoptosis in HL-60 cells. Our results showed that SB203580 (30mM) significantly inhibited p38 MAPK phosphorylation and SP600125 (30mM) also reduced JNK phosphorylation by more than fifty percent in HL(60 cells treated with 6mg/ml ATO compared to control cells [Fig. 4C-D]. Finally, TX(induced cytotoxicity changed expression levels of apoptotic proteins (Bax, cytochrome C, & Bcl-2), Annexin V, and PI in KG1a cells; leading to the apoptosis of APL cells coordinated by the phosphorylation MAPK signaling molecules [Fig.5].

4. DISCUSSION

It has been reported earlier that TX enters into the APL cells cytoplasm by a diffusion process through plasma membrane, and produces oxidative stress and clastogenic effect by generating reactive oxygen species [1, 7, 19]. Several scientific evidences suggest that

arsenic trioxide induces oxidative stress, causes cell cycle arrest, and forces cells to undergo apoptosis in a variety of cancer cells including leukemia cells [1,19,21–27]. Cytotoxicity is a complex process that usually involves oxidative stress, transcriptional factors and stress signaling leading to cell death [3]. Accumulating evidence from published data suggests that TX induces cytotoxicity alone or in combination with other compounds by various mechanisms in head and neck squamous cell carcinoma cells [30], small cell lung cancer cells [31], lung cancer cells [32], breast cancer cells [33], and prostate and ovarian carcinoma cells [42]. MAPK is a stress signaling molecule involved in cell cycle regulation and apoptosis in many cancer cells [43]. Few reports indicate that TX causes apoptosis through activation/modulation of MAPK signaling molecules in various cancer cells [28, 29,37–39]. However, the detailed molecular mechanisms of TX-induced cytotoxicity and MAPK signaling cascade in APL cell lines are poorly understood. Here, we report for the first time TX(induced cytotoxicity was associated with the inhibition of cycle progression, early and late apoptosis, and MAPK signaling cascade in APL cell lines. Our findings indicate that TX significantly induces cytotoxicity in all three APL cells in a concentration dependent manner (Fig.1A-C). Other research groups have reported that TX inhibits growth and proliferation of colon cancer cells and myeloma cells through cell cycle regulation/arrest [19, 21, 44]. We found that TX(induced cytotoxicity inhibits cell cycle progression in a concentration dependent manner in APL cells (Fig.2i-v). Several research groups including ours have reported that TX(induced oxidative stress causes apoptosis through activation of phosphatidylserine externalization, expression and translocation of apoptotic proteins, and caspase 3 level in cervical cancer cells, lymphoma B(cells, human B cells, and leukemia cells [1,7, 26, 27, 29, 45]. Our results show that TX – induced cytotoxicity stimulated the expression of Bax, cytochrome C, caspase 3, and down regulated the expression of Bcl-2 in KG1a cells (Fig. 3A). It also activated phosphatidylserine externalization (early apoptosis, annexin V binding) and genomic damage (late apoptosis, PI internalization) in APL cells in a concentration dependent manner (Fig.3 Bi-v). Published reports also indicate that TX(induced apoptosis is linked to c-jun-terminal kinase (JNK/SAP) and p38MAPK activation in cervical cancer cells [29] as well as NKM(1 cells [28]. It also causes autophagy through activation of Erk1/2 signaling pathway in U118-MG cells [46]. Our research data show that TX(induced cytotoxicity activated phosphorylation of P38MAPK, JNK, and Erk1/2 in HL-60 cells. Whereas, TX(induced cytotoxicity activated phosphorylation of P38MAPK and Erk1/2 and down regulated phosphorylation of JNK in KG1a cells (Figure 4A-B). Our specific MAPK pathway inhibitor studies also confirm the involvement of MAPK signaling pathway in TX-induced cytotoxicity in HL-60 cells (Figure 4C-D). Overall, TX(induced cytotoxicity inhibited cell cycle progression and changed the expression levels of apoptotic proteins leading to the induction of both early and late apoptosis coordinated through phosphorylation of MAPK molecules in APL cells [Fig.5].

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Abbreviations

ANOVA	One way analysis of variance
APL	Acute promyelocytic leukemia
TX	Arsenic trioxide (Trade name, Trisenox)
PI	Propidium Iodide
ATRA	All trans retinoic acids
DNA	Deoxyribonucleic acid
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N-terminal kinases
PBS	Phosphate buffer saline
ERK	Extracellular signal– regulated kinases

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Figure 1. Trisenox causes cytotoxicity in APL cells

APL cells were exposed to various concentrations (0, 2, 4, 6 & 8 µg/ml) of Trisenox (TX) for 24 hours and the LDH (lactate dehydrogenase) released was measured. Highly statistically significant increases ($p < 0.01$) in percentages of cytotoxicity were observed in TX treated HL- 60 [Fig.1A], KG-1a [Fig.1B] and NB4 [Fig.1C] cells, in a concentration - dependent fashion.

Figure 2. Trisenox inhibits cell cycle progression

KG1a cells were treated with different concentrations of TX for 24 hours. After incubation, cells were washed with PBS and immunocytochemistry and confocal imaging of ki67 were performed. Ki67 protein expression was significantly reduced in a concentration dependent manner [Fig.2i-v].

Figure 3. Trisenox induces apoptosis

KG1a cells were grown in presence or absence of different concentrations of TX for 24 hours. After incubation, cells were lysed in RIPA buffer and checked expression level of apoptotic proteins by western blotting. Bax, cytochrome C, and caspase 3 expression were activated, whereas Bcl-2 expression was reduced [Fig.3A]. Both control and TX treated cells were stained in mixture of annexin V and PI (propidium iodide) and imaged with CBA cellometer/confocal microscopy. Annexin V and PI expression levels were significantly increased in TX(treated cells in a concentration dependent [Fig.3Bi-v].

Figure 4. Trisenox modulates stress signaling, MAPK

Both control/untreated and TX treated cells were lysed in RIPA buffer and protein lysates were made. Phosphorylation level of MAPK molecules was analyzed by western blotting using phospho MAPK antibodies. TX modulated phosphorylation of P38MAPK, JNK, and Erk in both HL-60 and KG1a cells [Fig. 4A-B]. Both cells were further incubated for 30 minutes with P38MAPK and JNK inhibitors and analyzed by western blotting using phospho primary antibodies. MAPK inhibitors inhibited the phosphorylation of JNK and p38 MAPK molecules and confirmed its involvement in TX-induced cytotoxicity in APL cells [Fig.4C(D)].

Figure 5. Trisenox -induced cytotoxicity pathway

TX enters into APL cells through diffusion and induces cytotoxicity through inhibition of cell cycle progression, modulation of apoptotic proteins, annexin V, PI expression, and MAPK molecules phosphorylation [Fig.5].