Original Paper

Zinc Depletion by TPEN Induces Apoptosis in Human Acute Promyelocytic NB4 Cells

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Key Words

TPEN • Zinc • Nitric oxide • Apoptosis • Acute promyelocytic leukemia

Abstract

Background/Aims: The effects of zinc signaling on proliferation or apoptosis of leukemia cells remain elusive. In the present study, we used N, N, N', N'-tetrakis-(2-pyridylmethyl)-ethylene-diamine (TPEN), a membrane-permeable zinc chelator, to evaluate the effect of zinc depletion on survival and apoptosis of NB4 acute promyelocytic leukemia (APL) cells. **Methods:** The pro-apoptotic effects of TPEN on NB4 cells were examined by flow cytometry, and observed using an optical microscope. Intracellular labile zinc, nitric oxide (NO) or reactive oxygen species (ROS) changes caused by TPEN were measured by flow cytometry. We then explored possible roles of the crosstalk between intracellular labile zinc signaling and nitric oxide signaling in TPEN-triggered apoptosis. **Results:** we found that TPEN induced apoptosis in NB4 APL cells in a dosage-dependent manner. We further demonstrated that TPEN triggered apoptosis by attenuating intracellular zinc and nitric oxide signaling in NB4 cells. Both exogenous zinc supplement and the nitric donor sodium nitroprusside (SNP) pre-incubation reversed TPEN-mediated inhibition of intracellular NO and Zn²⁺ signaling, and rescued NB4 cells from apoptosis. **Conclusion:** These results suggest for the first time that crosstalk between zinc signaling and nitric oxide pathway is essential for the survival of NB4 cells. TPEN induces apoptosis in NB4 cells *via* negatively regulating intracellular NO and Zn²⁺ signaling. Our *in vitro* data suggest that zinc depletion by TPEN may be a potential therapeutic strategy for APL.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of leukemias that result from malignant transformation of hematopoietic precursors [1, 2]. Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia characterized by a t (15:17) translocation that yields a PML/RAR α fusion oncoprotein, which blocks maturation and apoptosis of neutrophils at the promyelocyte stage [3, 4]. Due to the tendency for severe bleeding in patients, APL was historically described as the most malignant form of acute leukemia with

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a fatal course of only weeks [5]. Introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in the treatment has largely improved the clinical outcomes of patients with APL [6, 7]. However, about 48% of the APL patients treated with ATRA may develop the ATRA-related differentiation syndrome [8]. Some side effects of ATO treatment exist, and the safety of long-term application of ATO is of great concern [9]. Furthermore, resistance or intolerance to these drugs is often encountered [10]. There are still some APL patients with poor clinical outcomes [11, 12]. Hence, new chemicals and strategies with therapeutic potential for APL are still of intense interest.

Zinc is vital for numerous cellular processes, including survival, proliferation, aging, apoptosis, autophagy, and differentiation, both in normal and cancer cells [13-16]. As a cofactor for an estimated 3,000 human proteins [17], aberrations in zinc homeostasis contribute to development and progression of human cancers [18, 19]. Previous studies are mainly focused on the cytotoxicity of excessive zinc supplement on cancer cells [20-22]. Recently, N, N, N', N'-tetrakis-(2-pyridylmethyl)-ethylene-diamine (TPEN), a membrane-permeable zinc chelator, shows pro-apoptotic effects on some cancer cells, indicating that zinc depletion may be a potential strategy for cancer treatment [23, 24]. However, zinc homeostasis has mainly been studied in solid tumor cells, accumulating only a limited wealth of zinc-related study in cancer biology. Although low serum levels of zinc have been noted in certain kinds of leukemia, the underlying reasons are rarely studied [25, 26]. The role of zinc homeostasis in survival or apoptosis in leukemia cells remains unclear.

Nitric oxide (NO) has both pro- and anti-apoptotic effects on cancer cells, mainly depending on its concentration [27-30]. Physiological level of NO signaling is essential for cell survival and proliferation, and depletion of NO inhibits proliferation and induces apoptosis in various cancer cells [31, 32]. Meanwhile, excessive NO triggers apoptosis of cancer cells with evaluated oxidative stress [33]. It has been reported that NO can promote Zn^{2+} release from intracellular storage and trigger neuronal cell death [34]. However, effects of the crosstalk between NO and Zn^{2+} signaling on cancer cells, especially on leukemia cells, remain to be explored.

In the present study, we investigated the effects of zinc depletion by TPEN on apoptosis of human acute promyelocytic NB4 cells. Pro-apoptotic effect exerted by TPEN on NB4 cells was observed and quantitatively determined by flow cytometry. The crosstalk between labile zinc signaling and nitric oxide signaling was then examined to elucidate its role in TPEN-mediated death of leukemia cells.

Materials and Methods

Cell culture and reagents

The NB4, Kasumi-3, THP-1 and U937 acute myeloid leukemia (AML) cell lines were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine (All from Thermo Fisher Scientific, Waltham, MA, USA), and maintained in a humidified incubator (37°C, 5% CO₂). TPEN, Zinc sulfate (ZnSO₄), N-acetylcysteine (NAC), Sodium nitroprusside (SNP), Carboxy-PTIO (C-PTIO), Dichloro-dihydro-fluorescein diacetate (DCFH DA) and Propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) and Zinpyr-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The pan-caspase inhibitor Z-VAD-FMK and the caspase-8 inhibitor Z-IETD-FMK were purchased from Selleck Chemicals (Houston, TX, USA). The pan-caspase inhibitor Q-VD-OPh was purchased from MedChem Express (Monmouth Junction, NJ, USA). The caspase-8 inhibitor Ac-IETD-CHO was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability analysis

After being stimulated with indicated concentrations of TPEN for 24 h, the viability of NB4 cells was measured using Cell Counting Kit-8 (CCK-8, Beyotime, Nantong, China) according to the manufacturer's instructions.

Cell cycle assay

After being fixed in 70% ethanol at -20°C overnight, cells were pelleted, washed with PBS twice, and then resuspended in propidium iodide (20 µg/ml) and RNase (200 µg/ml) -containing buffer. Following incubation at 37°C for 30 min in the dark, NB4 cells were analyzed by flow cytometry for cell cycle distribution.

Flow cytometric analysis for apoptosis

Cells were stimulated with TPEN with or without ZnSO₄, SNP, or C-PTIO for the indicated time periods. Cells were then harvested, washed with a Ca²⁺-containing binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, and 2.5 mM CaCl₂, pH=7.4) and then co-stained with Annexin V-EGFP and PI at room temperature for 20 min. PI was added just 5 min before flow cytometric analysis. Annexin V-positive/PI-negative, and AnnexinV-positive/PI- positive populations represent cells in early and late apoptosis respectively.

Measurement of intracellular reactive oxygen species (ROS) by flow cytometry

Intracellular ROS level was detected by flow cytometry using DCFH DA, according to the manufacturer's instructions.

Measurement of intracellular nitric oxide by flow cytometry

DAF-FM DA is a sensitive and cell-permeable fluorescent probe for the detection of intracellular NO production. About 1 × 10⁶ cells were harvested and loaded with 5 µM DAF-FM DA at 37°C in the dark for 20 min. Cells were then washed three times with PBS and analyzed by flow cytometry.

Measurement of intracellular labile Zn²⁺ by flow cytometry

Relative labile Zn²⁺ level in NB4 cells was measured as previously described [35]. Briefly, about 1 × 10⁶ cells were harvested and loaded with 5 µM Zinpyr-1 at 37°C in the dark for 20 min, washed three time with PBS, and then analyzed by flow cytometry.

Western blot

Total Proteins were extracted from NB4 cells with cell lysis buffer for western blotting and immunoprecipitation (Beyotime Institute of Biotechnology, Nantong, China), separated by 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature with 5% non-fat milk dissolved in TBST buffer, and then incubated with primary antibodies at 4°C overnight. Washed with TBST for three times (10 min for each time), the membranes were incubated with secondary antibodies for 1 h at room temperature. Another round of membrane washes with TBST were performed before chemiluminescent detection. Anti-Caspase 3, anti-cleaved-Caspase 3, anti-Caspase 8, and anti-Caspase 9 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-actin antibody was obtained from Abgent (San Diego, CA, USA).

Statistical analysis

All analyses were performed using GraphPad Prism software (Version 6.02, La Jolla, CA, USA). The results are expressed as mean values with standard deviations. Student's t-test, or one-way ANOVA test with Dunnett's post-hoc was performed as appropriate. Differences were considered statistically significant when *P* < 0.05.

Results

TPEN triggers apoptosis of NB4 APL cells in a dose- and caspase- dependent manner

We found that TPEN is cytotoxic to NB4 cells in a dose-dependent way using CCK8 screening (Fig. 1A). Exposure to 5 µM TPEN for 24 h was enough to induce an approximate reduction of 50% in cell viability. And almost 90% inhibition of NB4 cells was achieved when incubated with 25 µM TPEN for 24 h. These data suggest that TPEN induces growth arrest or triggers apoptosis of NB4 APL cells in a dose-dependent manner. We further observed cell morphology of NB4 cells exposed to TPEN under an inverted microscope, and characterized

apoptotic NB4 cells using dual staining of Annexin V and PI. Typical apoptotic morphology of NB4 cells, such as cell shrinkage or cytoplasmic vacuolar changes, was observed following treatment with TPEN for 24 h at 5 μ M (Fig. 1B). Annexin V/PI staining assay yielded similar results (Fig. 1C). Treated with 5 μ M TPEN for 24 h, activation and cleavage of caspase-3, -8, and -9 was detected (Fig. 1D). Cell cycle assay results suggest that NB4 cells treated with 5 μ M TPEN for 12 h arrested in G0/G1 stage in the initial stages of apoptosis (Fig. 1E). We further pre-treated NB4 cells with pan-caspase inhibitors or caspase-8 inhibitors before

Fig. 1. Zinc chelator TPEN suppresses the viability and induces apoptosis of NB4 APL cells. (A) NB4 cells were treated with indicated concentrations of TPEN (0–25 μ M) for 24 h, and then subjected to a CCK-8 assay for cell viability analysis. (B) Cell morphology was observed and recorded using an optical microscope. The scale bar represents 20 μ m. A partially enlarged view was shown in the lower left corner of every picture. (C) Apoptosis of NB4 cells treated with indicated concentrations of TPEN (0–25 μ M) was measured with Annexin V/PI staining by flow cytometry. Early apoptotic cells: Annexin V+/PI-; necrotic/late apoptotic cells: Annexin V+/PI+. (D) Western blot analysis of caspase-8, -9 and -3 expression in NB4 cells treated with TPEN (5 μ M) for indicated time periods (0–24 h). β -Actin served as a loading control. Experiments were performed in triplicates, and representative blots were shown. (E) NB4 cells incubated with TPEN (0–5 μ M) for 12 h were fixed and subject to cell cycle analysis by flow cytometry after PI staining. Living cells in the group treated with 5 μ M TPEN were gated for further analysis. Data are represented as mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.005 compared with the vehicle control.

Fig. 2. The pan-caspase inhibitors block TPEN-induced apoptosis of NB4 cells. NB4 cells were pre-treated with 50 μ M Z-VAD-FMK or 50 μ M Q-VD-OPh for 1 h, and then treated with 5 μ M TPEN for 24 h. (A) Cell morphology was observed and recorded using an optical microscope. The scale bar represents 20 μ m. A partially enlarged view was shown in the lower left corner of every picture. (B) Apoptosis rate was measured with Annexin V/PI dual staining by flow cytometry.

Fig. 3. The caspase-8 inhibitors do not rescue NB4 cells from TPEN-triggered apoptosis. NB4 cells were pre-treated with 50 μ M Z-IETD-FMK or 100 μ M Ac-IETD-CHO for 1 h, and then treated with 5 μ M TPEN for 24 h. (A) Cell morphology was observed and recorded using an optical microscope. The scale bar represents 20 μ m. A partially enlarged view was shown in the lower left corner of every picture. (B) Apoptosis rate was measured with Annexin V/PI dual staining by flow cytometry.

TPEN stimulation, and found that TPEN-triggered apoptosis was blocked by pan-caspase inhibitors (Fig. 2), not caspase-8 inhibitors (Fig. 3). These data indicated that TPEN mainly induced apoptosis via intrinsic pathway in NB4 APL cells. We also found that TPEN inhibited cell viability and induced apoptosis in another three AML cell lines, including Kasumi-3, THP-1 and U937 (Fig. 4 and Fig. 5).

TPEN induces apoptosis of NB4 cells with decreased intracellular ROS level

Oxidative stress plays important roles in regulating apoptosis of leukemia cells [36, 37]. Previous studies have demonstrated that elevated ROS are involved in TPEN-induced apoptosis of human acute lymphoblastic leukemia (ALL) cells [38]. In contrast, TPEN-induced apoptosis in NB4 APL cells associated with decreased intracellular ROS (Fig. 6A), and the widely used ROS scavenger NAC failed to rescue NB4 cells from TPEN-induced apoptosis (Fig. 6B and 6C).

Fig. 4. TPEN inhibits cell viability in Kasumi-3, THP-1 and U937 AML cells. Kasumi-3 (A), THP-1 (B) or U937 (C) cells were treated with indicated concentrations of TPEN (0–25 μM) for 24 h, and then subjected to a CCK-8 assay for cell viability analysis. All experiments were done at least in three independent replicates and data were shown as mean ± SD. *** $P < 0.005$.

Fig. 5. TPEN induces apoptosis in Kasumi-3, THP-1 and U937 AML cells. Kasumi-3, THP-1, and U937 cells were treated with indicated concentrations of TPEN (0–25 μM) for 24 h. (A) Cell morphology was observed and recorded using an optical microscope. The scale bar represents 20 μm. A partially enlarged view was shown in the lower left corner of every picture. (B) Apoptosis rate was measured with Annexin V/PI dual staining by flow cytometry.

TPEN induces apoptosis of NB4 cells through inhibiting intracellular nitric oxide (NO) signaling

The crosstalk between Zn^{2+} and NO signaling has been reported to play key roles in apoptosis of neurons [34]. We were interested in whether NO signaling regulates survival or apoptosis of NB4 APL cells during zinc depletion. Using the fluorescence probe DAF-FM DA, we found that TPEN induced apoptosis of NB4 APL cells with attenuated intracellular NO

Fig. 6. TPEN induces apoptosis of NB4 cells with decreased intracellular ROS. (A) NB4 cells treated with 5 μ M TPEN or vehicle were stained with DCFH-DA for ROS measurement. (B) Apoptosis rate of NB4 cells treated with TPEN with or without the presence of NAC was estimated by flow cytometry employing Annexin V/PI staining. (C) Cell morphology was observed and recorded using an optical microscope. The scale bar represents 20 μ m. A partially enlarged view was shown in the lower left corner of every picture. Data are represented as mean $\pm$ SD. *** P < 0.005 compared with the vehicle control.

signaling (Fig. 7A). Next, we treated NB4 cells with TPEN with or without SNP (a NO donor) or C-PTIO (a NO scavenger), and measured the intracellular NO level. It was found that SNP effectively reversed the decrease in NO caused by TPEN. In contrast, C-PTIO suppressed intracellular NO signaling with or without TPEN (Fig. 7A). Next, we further revealed that SNP pre-incubation rescued NB4 cells from TPEN-triggered apoptosis, whereas C-PTIO showed cytotoxicity to NB4 cells, and enhanced the pro-apoptotic effect of TPEN (Fig. 7B-D). Apoptosis rate was determined with Annexin V/PI staining by flow cytometry (Fig. 7B). Typical apoptotic signs of cellular morphology was observed under an optical microscope (Fig. 7C). Activation and cleavage of caspase-8, -9 and -3 was confirmed by western blot (Fig. 7D). These data suggest that TPEN induces apoptosis in NB4 cells at least partially *via* suppressing intracellular NO signaling.

Exogenous zinc supplement at 5 μ M has no obvious effect on intracellular NO signaling and survival of NB4 cells

To further scrutinize the crosstalk of intracellular labile zinc signaling and NO signaling on survival and apoptosis of NB4 APL cells, we first treated NB4 cells with 5 μ M zinc sulfate for 24 h in the absence of TPEN, and detected a slight increase in intracellular labile zinc signaling (Fig. 8A). No obvious changes in intracellular nitric oxide signaling or cell viability was observed (Fig. 8B-D).

Zinc supplement reverses reduction of intracellular NO signaling in NB4 cells in zinc depletion condition caused by TPEN

We further treated NB4 cells with 5 μ M TPEN with or without the presence of indicated concentrations of exogenous Zn^{2+} (0-5 μ M, pre-incubated for another 2 h) for 24 h, and detected intracellular NO level with DAF-FM DA by flow cytometry. We found that exogenous

Fig. 7. TPEN triggers apoptosis of NB4 cells through deregulating intracellular NO signaling. (A) NB4 cells were treated with 5 μ M TPEN in the presence or absence of SNP (500 μ M, pre-incubated for 2 h) or C-PTIO (50 μ M, pre-incubated for 2 h) for 24 h. Intracellular nitric oxide was monitored using the fluorescence probe DAF-FM DA. The results are represented as percentage of DAF-FM DA-positive cells. (B) Apoptosis was measured with Annexin V/PI dual staining by flow cytometry, and morphological changes of NB4 cells were observed and recorded using an optical microscope (C). The scale bar represents 20 μ m. A partially enlarged view was shown in the lower left corner of every picture. Western blot analysis was conducted to determine protein expression of caspase-3, caspase-8 and caspase-9 in NB4 cells treated with TPEN (5 μ M) in the presence or absence of SNP (500 μ M) or C-PTIO (50 μ M) for 24 h (D). All experiments were done at least in three independent replicates and data were shown as mean $\pm$ SD. * P < 0.05 and *** P < 0.005.

Zn²⁺ supplement reversed the reduction of NO signaling in TPEN-treated NB4 cells in a concentration-dependent manner (Fig. 9A-B).

Zinc supplement rescues NB4 cells from TPEN-triggered apoptosis

It has been previously reported that TPEN induces apoptosis in human ALL cells independently of its zinc-chelating ability [38]. To assess the role of zinc depletion in TPEN-triggered apoptosis, we measured the apoptosis rate in NB4 APL cells treated with TPEN with or without the presence of indicated concentrations of exogenous zinc. We demonstrated that exogenous zinc supplement efficiently reversed zinc depletion caused by TPEN and rescued NB4 cells from TPEN-triggered apoptosis in a dosage-dependent way (Fig. 10A-D). These data indicate that TPEN induces apoptosis of NB4 APL cells through its zinc-chelating activity.

The NO donor SNP reverses TPEN-triggered zinc depletion in a concentration-dependent manner

Nitric oxide has been reported to promote zinc release from its intracellular storage in neurons [39]. We are interested in the effects of intracellular NO signaling on Zn²⁺ signaling in NB4 APL cells during zinc depletion. Therefore, we treated NB4 cells with TPEN with or without SNP or C-PTIO at indicated concentrations, and measured intracellular labile Zn²⁺ level with the fluorescent sensor Zinpyr-1 by flow cytometry. We found that SNP reversed TPEN-triggered zinc depletion in a concentration-dependent way. In contrast, C-PTIO further aggravated zinc depletion caused by TPEN (Fig. 11). Our data suggest that NO signaling plays

Fig. 8. Exogenous zinc supplement at 5 μM has no obvious effects on intracellular nitric oxide signaling and apoptosis of NB4 cells. NB4 cells were treated with 5 μM zinc sulfate, and intracellular labile Zn^{2+} signaling was monitored using the specific fluorescent probe Zinpyr-1 by flow cytometry. Relative intracellular labile zinc level was expressed by the relative geometric mean fluorescence intensity of Zinpyr-1 (A). Apoptosis of NB4 cells treated with or without zinc sulfate was probed with Annexin V/PI dual staining by flow cytometry (B). The morphology of NB4 cells was observed and recorded using an optical microscope (C). The scale bar represents 20 μm . A partially enlarged view was shown in the lower left corner of every picture. Intracellular nitric oxide was monitored using the fluorescence probe DAF-FM DA. The results are represented as the percentage of DAF-FM DA-positive cells (D). Experiments were performed in triplicates. Data are represented as mean $\pm$ SD. * $P < 0.05$ compared with vehicle-treated cells.

Fig. 9. Attenuation of intracellular NO signaling caused by TPEN can be reversed by exogenous zinc supplement. NB4 cells were treated with 5 μM TPEN for 24 h with or without the presence of indicated concentrations of zinc sulfate (0–5 μM), intracellular nitric oxide was monitored using the fluorescence probe DAF-FM DA by flow cytometry (A). The results are represented as the percentage of DAF-FM DA-positive cells (B). Data were represented as mean $\pm$ SD of three independent experiments. ** $P < 0.01$ and *** $P < 0.005$.

important roles in regulating Zn^{2+} signaling and in zinc depletion-triggered apoptosis in NB4 APL cells.

Discussion

New targets and therapy strategies against leukemia are still of intense interest [40–42]. Zinc plays dual roles in regulating cell proliferation and apoptosis. *In vitro* cytotoxicity of zinc towards solid tumor cells, especially prostate [43] or breast cancer cells [44], has been widely reported. Recently, apoptosis induced by zinc depletion was also recognized as a potential therapeutic strategy against cancer [45, 46]. However, the role of zinc signaling in

Fig. 10. Exogenous zinc supplement reverses decrease in intracellular labile Zn^{2+} signaling and rescued NB4 cells from apoptosis caused by TPEN. (A) NB4 cells were pre-incubated with indicated concentrations of zinc sulfate (0–5 μM) for 2 h, and then treated with 5 μM TPEN for another 24 h. Relative labile zinc level was measured using the specific fluorescent probe Zinpyr-1 by flow cytometry, and expressed by the relative geometric mean fluorescence intensity of Zinpyr-1. Apoptosis was monitored with Annexin V/PI dual staining by flow cytometry (B). The morphology of NB4 cells was observed and recorded using an optical microscope (C). Scale bar: 20 μm . A partially enlarged view was shown in the lower left corner of every picture. (D) Western blot analysis of caspase-8, -9 and -3 expression in NB4 cells treated with TPEN (5 μM) alone or with the presence of zinc sulfate (2.5 μM) for 24 h. β -Actin served as a loading control. Experiments were performed in triplicates, and representative blots were shown. Data are represented as mean $\pm$ SD. * $P < 0.05$, *** $P < 0.005$ compared with vehicle-treated cells.

Fig. 11. The nitric oxide donor SNP attenuates TPEN-triggered zinc depletion status of NB4 cells. NB4 cells were treated with 5 μM TPEN for 24 h with or without indicated concentrations of SNP (0, 250 or 500 μM) or C-PTIO (0, 12.5, 25 or 50 μM), and then intracellular labile zinc was measured using Zinpyr-1 by flow cytometry. Labile Zn^{2+} level was represented as relative geometric mean fluorescence intensity of Zinpyr-1. Data were represented as mean $\pm$ SD of three independent experiments. ** $P < 0.01$, *** $P < 0.005$.

regulating survival or apoptosis of leukemia cells remains unclear. The work presented here demonstrates a pro-apoptotic effect of the zinc chelator TPEN on NB4 APL cells.

Hashemi et al. demonstrate that intracellular zinc depletion by TPEN induces apoptosis in breast cancer lines *via* the intrinsic pathway with the activation of caspase-9 not caspase-8 [47]. In the present study, although cleavage and activation of caspase-8, -9 and -3 were observed, TPEN-induced apoptosis in NB4 cells was blocked by pan-caspase inhibitors, but not by caspase-8 inhibitors. These data indicated that TPEN triggered apoptosis in NB4 APL cells mainly *via* the intrinsic pathway not the extrinsic pathway, consistently with TPEN-

induced apoptosis in breast cancer cells. It has been reported that caspase-8 activation is regulated by caspase-9 activation in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced Parkinson's disease [48]. Similarly, we believe that caspase-8 activation is most probably the by-product effect of caspase-9 activation in TPEN-triggered apoptosis in NB4 cells.

As a specific chelator of zinc, the pro-apoptotic effects of TPEN have been reported on several types of cells, including neuronal cells [49], prostate cancer cells [50], colon cancer cell [46], hepatocellular carcinoma cells [51] and breast cancer cells [52]. Mechanisms responsible for TPEN's cytotoxicity vary in different cancer cell lines. TPEN has been reported to induce apoptosis of colon cancer cells *via* excessive ROS production and DNA damage [46]. Hashemi et al. demonstrate that TPEN-triggered apoptosis in MCF-7 and MDA-MB468 breast cancer cells can be reversed by the ROS scavenger NAC [47]. Juriol et al. demonstrate that zinc deficiency induces NADPH oxidase-dependent superoxide anion production and increases antioxidant enzymes activity in cardiac tissue of male rats [53]. Recently, Mendivilperez et al. demonstrate that TPEN induces apoptosis of acute lymphoblastic leukemia (ALL) cells independently of its zinc chelating activity. Instead, it causes elevated oxidative stress [38]. In contrast, Matias et al. demonstrate that zinc chelation by TPEN mediates the lysosomal disruption and induces apoptosis without ROS production in human neuroblastoma SH-SY5Y cells [54]. It has also been reported that zinc overload increases ROS production and induces senescence in vascular smooth muscle cells [55]. These studies indicate that the responsiveness of ROS to zinc dyshomeostasis differs in different cells. As demonstrated here, we found that TPEN triggered apoptosis of human acute promyelocytic NB4 cells with decreased intracellular ROS levels. We further found that TPEN-triggered apoptosis in NB4 APL cells could not be reversed by NAC addition. Basal intracellular ROS and zinc level in NB4 cells might partially affect this process, but the specific details of decreased ROS production mechanism in response to TPEN exposure require further study. We also found that exogenous zinc supplement prevented TPEN-triggered apoptosis in NB4 APL cells, indicating the importance of its zinc-chelating activity in this process. These results suggest that TPEN may induce apoptosis of NB4 APL cells by a different mechanism from ALL cells.

Nitric oxide (NO) is another reactive molecule whose signaling has been shown to regulate survival and apoptosis in different cells [56, 57]. Zinc signaling has been reported to regulate iNOS expression and activity in keratinocytes [58] and aortic endothelial cells [59]. The crosstalk between NO and Zn^{2+} signaling in leukemia cells still remains to be elaborated. Although excessive NO is cytotoxic to cancer cells [27], physiological level of intracellular NO is essential for cell survival and proliferation [60]. Recently, Juriol et al. demonstrate that zinc deficiency activates apoptotic and inflammatory processes with decreased nitric oxide synthase activity in cardiac tissue of rats [53]. In the present study, we found that TPEN induces apoptosis of NB4 APL cells by downregulating intracellular NO signaling. We

Fig. 12. Schematic representation of the mechanism responsible for TPEN-induced apoptosis in NB4 APL cells.

further showed that exogenous zinc supplement reversed the attenuation in NO signaling and rescued NB4 cells from apoptosis under zinc depletion status, although zinc supplement at the same dosage has no obvious regulatory effects on intracellular NO signaling in normal NB4 cells in the absence of TPEN. All these results suggest that NO signaling plays protective roles in apoptosis of NB4 cells caused by zinc depletion.

Nitric oxide has been reported to promote the release of intracellular Zn^{2+} from zinc sulfur clusters in various cell types including lung fibroblasts [61], neurons [34], and endothelial cells [62]. In our study, we demonstrated that the NO donor SNP promoted intracellular labile zinc signaling in NB4 cells under zinc depletion status caused by TPEN, whereas the NO scavenger C-PTIO inhibited zinc signaling. In the absence of the zinc chelator TPEN, no significant regulatory effect of SNP or C-PTIO on labile zinc signaling in NB4 cells was detected. These results further verify the function of NO signaling in upregulating labile zinc signaling and reducing apoptosis in NB4 APL cells under zinc depletion status.

In summary, we reported the apoptogenic capability of the zinc chelator TPEN in NB4 APL cells, where its cytotoxicity is mediated by suppressing intracellular labile zinc signaling and NO signaling (Fig. 12). Our *in vitro* data suggest that zinc depletion by TPEN may be a potential therapeutic strategy against APL.

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Disclosure Statement

The authors declare no conflict of interest.

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