Myeloperoxidase expression as a potential determinant of parthenolide-induced apoptosis in leukemia bulk and leukemia stem cells

Yu Ri Kim, Ju In Eom, Soo Jeong Kim, Hoi Kyung Jeung, June-Won Cheong, Jin Seok Kim, and Yoo Hong Min

Department of Internal Medicine (Y.R.K., S.J.K., J.-W.C., J.S.K., Y.H.M.), Medical Research Center (J.I.E., H.K.J.), and Center for Chronic Metabolic Disease Research (Y.H.M.), Yonsei University College of Medicine, Seoul, Korea
MPO as determinant to PTL-induced apoptosis

Address correspondence to:
Yoo Hong Min, MD.
Department of Internal Medicine, Yonsei University College of Medicine,
250 Seongsanno, Seodaemun-gu, Seoul 120-752, Korea.
Tel: 82-2-2228-1930, Fax: 82-2-393-6884, E-mail: minbrmmmd@yuhs.ac

Number of text pages: 19
Number of tables: 2
Number of figures: 6
Number of references: 39
Number of words in the Abstract: 231
Number of words in the Introduction: 675
Number of words in Discussion: 1175
Abbreviations

7-AAD, 7-aminoactinomycin; ABAH, 4-aminobenzoic acid hydrazide; AML, acute myeloid leukemia; DCFH-DA, dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DPBS, Dulbecco’s phosphate buffered saline; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione S-transferase; HSC, hematopoietic stem cells; IKK, I kappaB kinase; JNK, c-Jun N-terminal kinase; LSC, leukemia stem cells; MMP, mitochondrial membrane potential; MPO, myeloperoxidase; NAC, N-acetylcystein; NOD/SCID, Non-obese diabetic/severe combined immunodeficient; NF-κB, nuclear transcription factor kappaB; PARP, poly(ADP)ribose polymerase; PE, phycoerythrin; PI, propidium iodide; PTL, parthenolide; ROS, reactive oxygen species; ScAc, succinylacetone; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; XIAP, X-linked inhibitor of apoptosis protein

Recommended section: Chemotherapy, Antibiotics, and Gene Therapy
Abstract

Since parthenolide (PTL) is an effective anti-leukemic agent, identifying molecular markers predicting response to PTL is important. We evaluated the role of myeloperoxidase (MPO) in determining the sensitivity of leukemia cells to PTL-induced apoptosis. In this study, the level of PTL-induced generation of reactive oxygen species (ROS) and apoptosis was significantly higher in the MPO-high leukemia cell lines compared to the MPO-low leukemia cell lines. Pretreatment of MPO-high leukemia cells with a MPO-specific inhibitor ABAH, or a MPO-specific small interfering RNA (siRNA) abrogated the PTL-induced ROS generation and apoptosis, indicating that MPO plays a crucial role in PTL-induced apoptosis in leukemia cells. PTL-induced apoptosis was accompanied by downregulation of NF-κB, Bcl-xL, Mcl-1, XIAP, and survivin, and selectively observed in primary acute myeloid leukemia (AML) cells expressing higher level of MPO (≥ 50%), while sparing both AML cells with lower MPO and normal CD34-positive (CD34+) normal bone marrow cells. The extent of PTL-induced apoptosis of the CD34+CD38- cell fraction was significantly greater in the MPO-high AML cases, as compared to the MPO-low AML (P<0.01) and normal CD34+ marrow cells (P<0.01). NOD/SCID human leukemia mice model also revealed that PTL preferentially targets the MPO-high AML cells. Our data suggest that MPO plays a crucial role in determining the susceptibility of leukemia cells to PTL-induced apoptosis. PTL can be considered a promising leukemia stem cell targeted therapy for AML expressing high level of MPO.
Introduction

Parthenolide (PTL) is a sesquiterpene lactone and the active component of the medicinal plant feverfew (*Tanacetum parthenium*) (Knight, 1995). PTL contains both an α-methylene-γ-lactone ring and an epoxide group, which interact with the nucleophilic sites of many biological molecules (Bork et al., 1997). In addition to its well-known anti-microbial and anti-inflammatory properties (Brown et al., 1997), PTL has been reported to inhibit cell growth and induce apoptosis in a variety of tumor cells (Wiedhopf et al., 1973; Woynarowski and Konopa, 1981; Ross et al., 1999; Zhang et al., 2004b). Importantly, PTL has also been shown to effectively eradicate acute myeloid leukemia (AML) stem cells and progenitor cells *in vitro*, while sparing normal hematopoietic stem cells (Guzman et al., 2005). Thus, PTL could be an attractive agent for treating myeloid leukemia bulk and leukemia stem cells (LSC).

Several mechanisms have been shown to be involved in the anti-tumor effect of PTL, including the inhibition of nucleic acid synthesis (Woynarowski and Konopa, 1981), induction of mitochondrial dysfunction (Wen et al., 2002), disruption of intracellular calcium equilibrium (Zhang et al., 2004b), induction of G2-M phase arrest (Wen et al., 2002; Pozarowski et al., 2003), and sustained activation of c-Jun N-terminal kinase (JNK) (Nakshatri et al., 2004; Zhang et al., 2004a). In addition, PTL blocks signal transduction and the activators of transcription 3 (STAT3) phosphorylation (Sobota et al., 2000). PTL is a potent inhibitor of NF-κB activation through direct binding to IκB-kinase (IKK) (Hehner et al., 1999; Kwok et al., 2001; Zhang et al., 2004a). Most importantly, PTL induces cell death through the generation of intracellular reactive oxygen species (ROS) in a glutathione-sensitive manner (Wen et al., 2002; Zhang et al., 2004b; Guzman et al., 2005). Since malignant cells are highly susceptible to PTL-induced cell death (Wang et al., 2006), it is important to identify specific markers which indicate the susceptibility of leukemic cells to PTL-induced cell death in order to allow the effectiveness of PTL as an anti-leukemia agent to be predicted. Myeloperoxidase (MPO), a heme protein abundantly expressed in azurophilic granules (Eiserich et al., 2002), catalyzes the formation of hypochlorous acid, a potent oxidant implicated in killing bacteria.
The MPO tissue destruction occurs through induction of necrosis and apoptosis (Wagner et al., 2000; Klebanoff, 2005). Expression of the MPO molecule is specific for myeloid precursors and their leukemic counterparts. Evidence of the functional significance of the MPO molecule, in addition to its diagnostic importance, in myeloid leukemia cells is accumulating. The percentage of MPO-positive blast cells is highly associated with clinical outcomes of AML (Takubo et al., 1983; Matsuo et al., 2003). The JALSG study demonstrated that AML patients with 50% or higher MPO-positive blast cells have a significantly better outcome (Matsuo et al., 2003), although the biological significance of the MPO molecule as a prognosis-determining factor in AML is unknown. Recently, it has been shown that MPO is a key regulator of oxidative stress-mediated apoptosis in myeloid leukemia cells (Nakazato et al., 2007). ROS is the direct mediator of green tea polyphenol(-)-epigallocatechin-3-gallate (EGCG)-induced apoptosis in MPO-positive leukemia cells, whereas EGCG fails to induce apoptosis in MPO-negative leukemia cells (Nakazato et al., 2007). Therefore, MPO may be an important determinant of myeloid leukemia cell sensitivity to oxidative stress-mediated apoptosis induced by several antileukemia agents.

The aim of this study is to evaluate whether MPO plays a critical role in PTL-induced apoptosis of leukemia cells. Therefore, the detailed molecular mechanism of PTL-induced apoptosis in relation to MPO expression was further investigated. Interestingly, the high susceptibility of leukemia cells to PTL-induced apoptosis is selectively documented in primary AML bulk cells, as well as CD34+CD38- AML LSC candidates expressing high levels of MPO. In contrast, PTL at a concentration inducing a substantial degree of apoptosis in the MPO-highly expressing leukemia cells does not affect survival of CD34-positive normal hematopoietic stem cells (HSC). According to our findings, MPO expression is a critical determinant of PTL-induced apoptosis in leukemia cells. Thus, PTL can be considered as a promising LSC-targeted therapy for AML with a high level of MPO.
Materials and Methods

Cells, reagents and culture

U937, K562, HL60, Kasumi-1, NB4, KG1, OCI-AML3 and MOLM13 human leukemia cell lines (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 supplemented with 25 mM L-glutamine (Cambrex BioScience, Walkersville, MD) and 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Primary leukemic cells were isolated of bone marrow samples of untreated patients with AML. The isolation of CD34-positive cells from normal bone marrow was performed using the immunomagnetic microbead method (Kim et al., 2007). Specimens were collected under the Severance Hospital Institutional Review Board–approved clinical sample procurement protocols, and informed consent was obtained in accordance with the Declaration of Helsinki. Parthenolide (4xi-Germacra-1(10),11(13)-dien-12-oic acid, 4,5-epoxy-6.alpha.-hydroxy-, gamma-lactone/ C15H20O3, Calbiochem, San Diego, CA) was reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 50 mM. Cells were resuspended at a density of $2 \times 10^5$/ml in a medium containing 10% FBS and incubated at 37°C in the presence of various concentrations of PTL for the indicated times, which were then harvested for subsequent analyses. Control cells were treated with equal amounts of the solvent. The caspase inhibitor zVAD-fmk (Benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone), an MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH), and a p38 inhibitor SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine) were purchased from Calbiochem. A heme biosynthesis inhibitor succinylacetone (ScAc) and JNK inhibitor SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one) were obtained from Sigma (St. Louis, MO) and Biomol (Plymouth Meeting, PA), respectively. All experiments were performed in triplicate.

Antibodies

Rabbit anti-MPO polyclonal and mouse anti-α-tubulin monoclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against
poly(ADP)ribose polymerase (PARP), XIAP, BID, tBID, Bak, PUMA, JNK, phospho(p)-JNK, p38, p-p38, survivin, as well as horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG, were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against NF-κB (p65), Mcl-1, and Bcl-xL were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal antibodies against caspase-8, capase-9, cytochrome-C, Smac/DIABLO, and rabbit polyclonal antibody against caspase-3 were obtained from PharMingen (San Diego, CA). To analyze human cell engraftment in the NOD/SCID experiments, bone marrow cells were blocked with the anti-Fc receptor antibody 2.4G2 and labeled with antihuman CD45 and CD33 antibodies (Becton Dickinson Biosciences, San Jose, CA).

Evaluation of MPO expression

MPO expression was evaluated using flow cytometry. For fixation and permeabilization of the cells, an Intraprep cell permeabilization kit (Beckman Coulter, Fullerton, CA) was used. The cells (5 × 10^5) were incubated for 30 minutes at room temperature with an anti-MPO antibody (Abcam, Cambridge, UK), and an isotype-matched mouse IgG served as the control. The cells were washed in cold DPBS and resuspended in 5µl FITC-labeled goat anti-mouse IgG antibody (PharMingen) for 15 minutes. To analyze MPO expression in CD34+CD38- AML cells, cells were labeled with anti-CD34-phycoerythrin (PE, Becton Dickinson), anti-CD38-PerCP-Cy5.5 (PharMingen), and anti-MPO antibody for 30 minutes. The cells were washed in cold DPBS (Dulbecco’s phosphate buffered saline) and resuspended in 5µl FITC-labeled goat anti-mouse IgG antibody. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS, San Jose, CA). The percentage of cells expressing MPO was defined on the gates set for the CD34+CD38- population.

Measurement of MPO activity

Cells were harvested, washed with DPBS and then homogenized in a phosphate buffer containing 1% hexadecyltrimethylammonium hydroxide and centrifuged at 4,500 × g for 15 minutes
at 4°C to assay for MPO in the supernatants. MPO activity was measured using an enzyme immunometric assay kit for human MPO (Assay Designs, Ann Arbor, MI).

**Stable transfection of K562 cells with wild-type or mutant MPO cDNA**

For transfection of wild-type or mutant MPO cDNA to K562 cells, the Nucleofection method was used (Kim et al., 2007). A transfection mixture was prepared by mixing 5 µg DNA with 100 µl human Nucleofector V solution (Amaxa Biosystems, Gaithersburg, MD). pCI-MPO wild-type and pCI-MPO/R569W mutant cDNAs were kindly provided by Dr. Yasushi Miyazaki (Nagasaki University, Japan), with the pCI empty vector was used as a control. The cell suspension (2 × 10⁶ cells/ml) was immediately electroporated using a Nucleofector instrument (program T-16; Amaxa Biosystems). After electroporation, cells were immediately cultured with a complete medium in 6-well plates in a 37°C/5% CO₂ incubator. Stable transfectants (K562/control, K562/MPO, and K562/R569W) were selected as a single clone in the presence of 1 mg /ml G418 (Sigma).

**Transient transfection of MPO siRNA**

MPO-specific small interfering RNA (siRNA) (5’-AGGCATCACCACCGTGCTAA-3’) was purchased from Qiagen, Inc. (Valencia, CA). The cell suspension of 2×10⁶ leukemia cells was immediately transfected with MPO-specific siRNA using the Nucleofector instrument (Amaxa Biosystems) in accordance with the manufacturer’s instructions. Immediately after electroporation, the cells were resuspended in a complete medium and incubated in a humidified 37°C/5% CO₂ incubator. Mock control siRNA transfection was also performed for control experiments. The cells were harvested after 24 hours and used in subsequent experiments.

**Apoptosis assay**

The Annexin V assay was performed, as described (Kim et al., 2007). Cultured cells were washed and incubated in 100 µl binding buffer containing 5 µl Annexin V-FITC (PharMingen). The nuclei were counterstained with propidium iodide (PI), and the fraction of apoptotic cells was
determined by flow cytometry. To identify apoptotic CD34+CD38- cells, cells were labeled with anti-
CD34-PE-texas red (ECD; Beckman Coulter) and anti-CD38-PE (BD Biosciences) for 30 minutes.
The labeled cells were subsequently resuspended in 100 µl Annexin V-binding buffer and incubated
with Annexin V-FITC and 7- aminoactinomycin (7-AAD, Beckman Coulter) for 20 minutes at room
temperature before analysis by flow cytometry.

Assessment of loss of mitochondrial membrane potential (MMP)

The loss of MMP was monitored using DiOC6 (Kim et al., 2007). For each condition, $4 \times 10^5$
cells were incubated at 37°C for 15 minutes in 1 ml of 40 nM DiOC6 (Calbiochem) and subsequently
analyzed by flow cytometry. The control experiments documenting the loss of MMP were performed
by exposing the cells to 5 µM carbamoyl cyanide m-chlorophenylhydrazone (Sigma), an uncoupling
agent that abolishes MMP.

Cell cycle analysis

Cells were fixed in 70% ethanol at -20°C for 16 hours and resuspended in 1mL of cell cycle
buffer (0.38 mM/l sodium citrate, 0.5 mg/ml RNase A, 0.01 mg/ml PI) at a concentration of $1 \times 10^6$
cells/ml. Cell cycle analysis was carried out using a FACSCalibur flow cytometer equipped with
Modfit LT 3.0 software (Verity Software House, Topsham, ME).

Measurement of intracellular generation of ROS

To assess the intracellular production of ROS, cells were treated with PTL alone or together
with the antioxidant N-acetylcysteine (NAC; (R)-2-acetamido-3-sulfanylpropanoic acid, Sigma).
After the treatment, the cells were incubated with 10 µM/l dichlorodihydrofluorescein diacetate
(DCFH-DA; Molecular Probes, Eugene, OR), which is oxidized by cellular ROS and forms the
fluorescent compound dihydrofluorescein. In addition, $1 \times 10^5$ cells were stained with 10 µM/l DCFH-
DA for 30 minutes at 37°C and then washed and resuspended in DPBS. The oxidative conversion of
DCFH-DA to dihydrofluorescein was measured by flow cytometry.
Measurement of intracellular GSH content

Changes in total cellular glutathione (GSH) levels were determined using a Glutathione Assay Kit (Sigma), in accordance with the manufacturer’s instructions. PTL-treated cells were harvested and lysed with a 1× lysis solution. Supernatant was added to each well of a 96-well plate, followed by the addition of 5µl glutathione S-transferase (GST), 2.5 µl substrate solution, and assay buffer. After thorough mixing and incubation at 37°C for 60 minutes, fluorescence emissions at 478 nm were determined, following excitation at 390 nm using a Luminescence Spectrometer (PerkinElmer Inc, Waltham, MA).

Western blot analysis

After the cells were lysed in lysis buffer (50 mM Tris-HCl (pH7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) and briefly sonicated, mitochondrial and cytosolic fractionation were performed using a Cytosolic/Mitochondria Fractionation Kit (Oncogene Research Products, San Diego, CA). Protein yields were quantified using the Bio-Rad detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Equivalent amounts of protein (20 µg) were boiled for 10 minutes and resolved on a 15% polyacrylamide gel by SDS PAGE. The proteins were subsequently transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), which were washed and incubated with the primary antibody for 2 hours at room temperature. After washing, the membranes were incubated with the relevant HRP-conjugated secondary antibody (1:3,000 diluted in TBST containing 5% bovine serum albumin) for 1 hour. After the membrane was washed four times in TBST, the reactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences).

NOD/SCID human leukemia mice model assay

Non-obese diabetic/severe combined immunodeficient (NOD/SCID; NOD.CB17-Prkdc scid/J) mice (Jackson Laboratories, Bar Harbor, ME) were irradiated with 2.7 Gy using a RadSource
X-ray irradiator (RadSource, Boca Raton, FL) a day prior to the transplantation. Human leukemia cells to be assayed were injected using the tail vein (5-10 × 10⁶ cells) in a final volume of 0.2 mL PBS containing 0.5% FBS. After 6 to 8 weeks, the animals were sacrificed, and their bone marrow was analyzed for the presence of human cells by flow cytometry.

**Statistical analysis**

Primary AML cases were divided into two groups based on the expression level of MPO protein in the leukemia cells: MPO-low and MPO-high. We arbitrarily defined primary AML cases as MPO-high and MPO-low AML according to whether the fraction of leukemic blasts expressing MPO was greater and lesser than 50% of the total mononuclear cells, respectively. The comparisons among the characteristics between groups were made using the χ² test for binary variables and the Mann-Whitney test for continuous variables. The statistics were calculated using SPSS software, version 11.0.1 (SPSS, Inc., Chicago, IL), with P < 0.05 considered as significant.
Results

MPO expression and activity in leukemia cells

MPO expression was examined in several leukemia cell lines using flow cytometric analysis. While MPO expression was observed to be low in the K562, U937, KG1 and OCI-AML3 cell lines, MPO was highly expressed in the HL60, NB4, Kasumi and MOLM13 cell lines (Fig. 1A, upper). This was confirmed by western blot analysis as the heavy (α)-subunit of mature MPO was observed in the HL60, NB4, Kasumi and MOLM13 cell lines but not in the K562, U937, KG1 and OCI-AML3 cell lines (Fig. 1A, lower). In addition, as shown in Fig. 1B, MPO activity was also remarkably higher in the HL60, NB4, and Kasumi cell lines as compared to both the K562 and U937 cell lines.

PTL-induced apoptosis according to MPO expression

Apoptosis of leukemia cells induced by various concentrations of PTL was measured. The sensitivity of leukemia cell lines to PTL-induced apoptosis was highly dependent on the level of constitutive MPO expression. A limited degree of apoptosis was observed in U937 (9.7 ± 0.2%), K562 (11.0 ± 0.1%), KG1 (6.2 ± 1.1%) and OCI-AML3 (1.8 ± 0.1%) after 24 hours of treatment with 10 μM PTL (Fig. 1C). However, the proportion of apoptotic cells in the HL60, NB4, Kasumi and MOLM13 cell lines was markedly increased in a dose-dependent manner with PTL treatment. After 24 hrs of treatment with 10 μM PTL, the mean proportion of apoptotic cells was 92.5 ± 0.7% in HL60, 81.5 ± 1.1% in NB4, 89.2 ± 0.8% in Kasumi and 73.2 ± 1.9% in MOLM13 cell lines which was remarkably higher than in the K562, U937, KG1 and OCI-AML3 cells (HL60 vs U937 or K562 or KG1 or OCI-AML3, p<0.001; NB4 vs U937 or K562 or KG1 or OCI-AML3, p<0.001; Kasumi vs U937 or K562 or KG1 or OCI-AML3, p<0.001; MOLM13 vs U937 or K562 or KG1 or OCI-AML3, p<0.001, Fig. 1C, left). This findings indicate that leukemia cells that express higher MPO levels are more sensitive to PTL-induced cell death. Significant increases in the proportion of leukemia cells in the sub-G1 fraction after PTL treatment were limited to the high MPO expressing cell lines (Fig. 1C right).
The extent of MMP disruption induced by PTL was assessed by measuring the mitochondrial-mediated uptake of a membrane potential-sensitive dye DiOC<sub>6</sub>. The loss of MMP was significantly greater in HL60 cells compared to U937 cells (Fig. 1D, upper), suggesting that mitochondrial-mediated apoptotic cell death induced by PTL occurred preferentially in leukemia cells that express higher level of MPO. PTL-induced cytoplasmic translocation of cytochrome-C and Smac/DIABLO (Fig. 1D, lower), as well as the cleavage of caspase-3, -8, -9 and PARP (Fig. 1E, upper), were also limited to HL60 cells. In contrast, cytoplasmic translocation of cytochrome-C and Smac/DIABLO and cleavage of caspase cascades in U937 cells did not occur following PTL treatment. Pretreatment of HL60 cells with 20μM of a pan-caspase inhibitor, z-VAD-fmk, led to the abrogation of PTL-induced cell death (Fig. 1E, lower). These results indicate an association between expression of MPO and susceptibility to PTL-induced apoptosis, being this mechanism caspase-dependent.

**PTL-induced ROS generation in relation to MPO expression**

In order to explore whether MPO is critically involved in PTL-induced oxidative damage in leukemia cells, PTL-induced generation of intracellular ROS was measured in various leukemia cell lines. Five hours after treatment with 10 μM PTL, flow cytometric analysis showed that the mean relative ROS levels in the MPO-high HL60, NB4 and Kasumi cells were remarkably higher than those in the MPO-low K562 and U937 cells (Fig. 2A, left). This finding suggests that MPO-high cells produce more ROS than MPO-low cells with PTL treatment.

When leukemia cells were preincubated with 1 mM of the free radical scavenger L-N-acetylcystein (L-NAC) an hour prior to PTL treatment, ROS generation (Fig. 2A, right), as well as apoptosis (Fig. 2B), was almost completely abrogated in HL60, NB4, and Kasumi cells. This abrogation clearly suggests that an elevated ROS is crucial for PTL-induced apoptosis in MPO-high leukemia cells. We also measured the alteration of GSH levels upon PTL treatment and significant GSH depletion was observed only in HL60, NB4, and Kasumi cells (Fig. 2C). In addition, L-NAC pretreatment of the MPO-high cell lines completely abrogates the PTL-induced GSH depletion, which

14
did not occur in the MPO-low cell lines (Fig. 2C).

In order to elucidate the role of MPO in ROS-mediated apoptosis that is induced by PTL, we evaluated the effect of the MPO-specific inhibitor ABAH and the heme biosynthesis inhibitor ScAc on PTL-induced apoptosis by flow cytometric analysis (Fig. 2D, left). Preincubation of NB4 cells with 100 μM ABAH or 0.5 mM ScAc resulted in a significant inhibition of PTL-induced cell death (Fig. 2D, right). We further evaluated the effects of MPO-specific siRNA-mediated knockdown of MPO expression on the PTL-induced cell death. Western blot analysis demonstrated that MPO expression was effectively downregulated in NB4 cells 24 hours after transfection of cells with a MPO-specific siRNA, as described in Materials and methods (Fig. 2E, upper). MPO silencing significantly decreased the level of PTL-induced apoptosis in NB4 cells in a dose-dependent manner, whereas it was not observed in the control siRNA-transfected NB4 cells (Fig. 2E, lower). Discernible effects were not seen in U937 cells. These findings clearly indicate that MPO activity plays a crucial role in PTL-induced cell death in leukemia cells.

**PTL-induced changes in the apoptosis-regulating molecules according to MPO expression**

Alterations in the levels of various anti-apoptotic and pro-apoptotic molecules were evaluated following PTL treatment in leukemia cells. MPO-high HL60 cells showed a marked decrease in the levels of NF-κB (p65), Bcl-xL, Mcl-1, XIAP, and survivin after 10 μM PTL treatment for 24 hours (Fig. 3A). In contrast, these anti-apoptotic molecules were not affected by PTL in MPO-low U937 cells. BID cleavage, Bak induction, and the presence of PUMA were observed only in HL60 cells with PTL treatment (Fig. 3A). A greater extent of c-Jun N-terminal kinase phosphorylation (p-JNK) was also observed in HL60 cells (Fig. 3B, upper). However, p38 MAPK and phospho-p38 MAPK were altered neither in U937 nor HL60 cells. Preincubation of HL60 cells with 10 μM JNK-specific inhibitor SP600125 for 3 hours abrogated PTL-induced cell death (Fig. 3B, lower), suggesting that PTL-induced cell death in HL60 cells occurs through the JNK pathway. However, pretreatment of HL60 or U937 cells with the p38 specific inhibitor SB203580 did not affect PTL-induced cell death (Fig. 3B, lower).
Induced overexpression of wild-type MPO-enhances PTL-induced apoptosis

To determine whether MPO expression directly contributes to the sensitivity of leukemia cells to PTL-induced apoptosis, two stable transfected cell lines that overexpress wild-type MPO (K562/MPO) or mutant MPO (K562/R569W) were used. K562 cells transfected with a pCI empty vector were used as a control. As shown by the flow cytometric and western blot analysis, the heavy (α) subunit MPO protein was highly expressed in K562/MPO cell line, although not in the parental K562, K562/control, or K562/R569W cell lines (Fig. 4A).

The percentage of apoptotic cells in K562/MPO cells (65.8 ± 7.6%) 24 hrs after treatment with 10 μM PTL was found to be significantly higher as compared to parental K562 (5.7 ± 1.2%, p<0.05), K562/control (6.1 ± 1.3%, p<0.05), and K562/R569W cells (11.4 ± 2.8%, p<0.05) (Fig. 4b1). The histograms show a greater extent of sub-G1 populations in K562/MPO cells after PTL treatment (Fig. 4b2, left). The proportion of cells at the sub-G1 phase was also significantly higher in K562/MPO (58.9 ± 0.1%) when compared to K562 cells (3.5 ± 0.7%, p<0.01; Fig. 4b2, right). The loss of MMP observed 24 hrs after PTL treatment was also significantly higher in K562/MPO cells (45.0 ± 8.7%) than in K562 cells (0.7 ± 0.1%, p<0.05, Fig. 4b3, left). PTL-induced cytoplasmic translocation of cytochrome-C and Smac/DAIBLO was observed only in K562/MPO cells (Fig. 4b3, right). Caspase-dependent apoptosis, which was abolished with 20 μM z-VAD-fmk pretreatment prior to PTL addition, was confined to K562/MPO cells (Fig. 4b4). These findings suggest that MPO plays an important role in determining the sensitivity of myeloid leukemia cells to PTL-induced apoptosis through a mitochondria-mediated, caspase-dependent manner.

Pretreatment of K562/MPO cells with L-NAC pretreatment completely inhibited the PTL-induced ROS generation, indicating that elevated ROS is crucial for PTL-induced apoptosis in K562/MPO cells (Fig. 4C, upper). PTL-induced ROS generation was also significantly higher in the K562/MPO cells (31.5 ± 1.0%) compared with parental K562 cells (2.4 ± 0.3%, p<0.01) (Fig. 4C, lower). Likewise, MPO overexpression contributed to PTL-induced GSH depletion. GSH depletion was significantly greater in the K562/MPO cells compared to K562 cells (p<0.001, Fig. 4D). In contrast to the parental K562 cells, treatment of K562/MPO cells with PTL for 24 hours resulted in a
marked decrease in the level of NF-κB, Bcl-xL, Mcl-1, XIAP, and survivin, indicating that MPO expression is directly associated with PTL-induced down-regulation of several anti-apoptotic molecules (Fig. 4E). JNK phosphorylation was also more prominent in K562/MPO.

Differential sensitivity of primary AML cells to PTL according to MPO expression

We examined primary leukemic blasts obtained from patients with de novo AML and evaluates the correlation between the extent of PTL-induced cell death and their MPO expression levels (Table 1). Although MPO is an enzyme that is associated with myeloid lineage leukemia, variations in the proportion of MPO-positive leukemia cells were observed among the cases. As described in the Statistical analysis, primary AML cases were arbitrarily defined as the MPO-high and MPO-low AML according to whether the fraction of leukemic blasts expressing MPO was greater or less than 50% of the total mononuclear cells. It was found that MPO activity was significantly higher in cases classified as MPO-high AML compared to MPO-low AML (p<0.01) and normal CD34+ bone marrow cells (p<0.01) (Fig. 5A). The average percentage of apoptotic cells induced by PTL treatment was also significantly higher in MPO-high AML cases (44.4 ± 0.6%, n = 6), compared to MPO-low AML cases (1.4 ± 0.7%, n = 4, p<0.001) and normal bone marrow CD34+ cells (8.8 ± 0.5%, n=4, P<0.001) (Fig. 5B). The representative histograms show a greater extent of ROS production in MPO-high AML cells (Fig. 5C, upper). PTL-induced increase in the relative ROS levels was also significantly higher in the MPO-high AML (24.4 ± 1.6%) compared with MPO-low AML cases (5.9 ± 0.3%, p<0.001) and normal bone marrow CD34+ cells (5.6 ± 0.6%, p<0.001, Fig. 5C, lower). Additionally, a significant level of GSH depletion was observed only in MPO-high AML (Fig. 5D). As a whole, these findings suggest that PTL is a potent anti-leukemic agent which potentially induce ROS generation and apoptosis in primary MPO-high AML cells, while sparing normal hematopoietic stem cells and MPO-low AML cells.

MPO expression and PTL-induced apoptosis in CD34+CD38- LSC

MPO expression in potential LSC candidate CD34+CD38- leukemic blasts from 14
untreated AML patients (supplementary data1), were quantitatively measured based on the gated CD34+CD38- cell population by flow cytometry analysis. Interestingly, MPO expression was significantly higher in CD34+CD38- cells obtained from MPO-high AML cases than CD34+CD38- cells obtained from the MPO-low AML cases and normal bone marrow CD34+ cells (Fig. 6A, upper, middle, left lower). The proportion of the MPO-positive CD34+CD38- cells in the MPO-high AML sample (80.2 ± 5.5%) was significantly higher than that in the MPO-low AML sample (3.6 ± 1.0%, p<0.001) and the normal bone marrow CD34+ cells (3.1 ± 1.7%, p<0.001) (Fig. 6A, right lower). The extent of apoptosis in CD34+CD38- leukemia cells induced by PTL treatment was also significantly greater in MPO-high AML cases (87.3 ± 2.7%) compared to the MPO-low AML cases (32.5 ± 6.4%, p<0.01) and the normal bone marrow CD34+ cells (26.0 ± 11.5%, p<0.01) (Fig. 6B).

**NOD/SCID human leukemia mice model**

The NOD/SCID human leukemia mice model was used in order to further evaluate PTL-induced ablation of leukemia stem cells in relation to MPO expression. Primary leukemia cells were obtained from MPO-high AML and MPO-low AML cases, while CD34-positive bone marrow cells were obtained from healthy donors. These cells were preincubated with 10 μM PTL for 24 hours and transplanted into sublethally irradiated NOD/SCID mice. After 6 to 8 weeks, bone marrow cells were harvested and labeled with anti-human CD45 antibody to determine the percentage of human cells that were engrafted in each animal. As shown in Fig. 6C, PTL treatment significantly reduced the engraftment ability of primary leukemia cells that were harvested from the MPO-high AML cases, compared to untreated MPO-high AML control cells. However, the engraftment potential of the PTL-treated primary leukemic blasts from the MPO-low AML cases was not decreased when compared to the untreated control cells. The engraftment potential of normal CD34+ bone marrow cells did not change with PTL treatment in the NOD/SCID human leukemia mice model (Fig 6C). As a whole, these results suggest that PTL is able to ablate the MPO-expressing AML bulk and LSC while sparing the normal functioning CD34+ hematopoietic progenitor cells.
Discussion

PTL can be considered an attractive anti-leukemia agent, as it effectively eradicates AML cells, as well as AML LSC in vitro, while sparing normal hematopoietic stem cells (Guzman et al., 2005). In this study, we found the expression of MPO to be a crucial determinant of sensitivity to PTL-induced cell death in both myeloid leukemia cells and CD34+CD38- LSC candidates. The susceptibility of leukemia cells to PTL-induced apoptosis was significantly higher in the MPO-high leukemia cells and in the CD34+CD38- LSC obtained from MPO-high AML cases. The results also demonstrated that PTL does not affect the survival of the CD34+ normal bone marrow cells when used at a concentration that induces a substantial degree of apoptosis in the MPO-high leukemia cells. These findings suggest that PTL treatment may be a promising targeted therapy for MPO-high AML.

It was shown that PTL-mediated anticancer effects may be related to increased generation of ROS in cancer cells (Kwok et al., 2001; Wen et al., 2002; Wang et al., 2006; Pajak et al., 2008). ROS induces apoptosis in a variety of cancer cells by stimulating proapoptotic signaling molecules, activating the p53 protein pathway, or initiating the mitochondrial apoptosis pathway (Wen et al., 2002; Zhang et al., 2004a; Zhang et al., 2004b), in a GSH-sensitive manner (Zhang et al., 2004b; Pajak et al., 2008). Therefore, the identification and attenuation of molecules that control the generation of ROS in leukemia cells could enhance their susceptibility to PTL-induced apoptosis.

In this study, we demonstrated that PTL remarkably induces the generation of ROS and apoptosis specifically in both MPO-high leukemia cell lines and MPO-high primary leukemic cells obtained from AML patients. However, PTL failed to generate ROS or induce apoptosis in MPO-low leukemia cells. Pretreatment of leukemia cells with NAC abrogated PTL-induced ROS generation, GSH depletion and cell death only in the MPO-high cells, suggesting that the generation of oxidant species is associated with PTL-induced cell death in the MPO-high leukemia cells. The MPO-specific inhibitor ABAH and the heme biosynthesis inhibitor ScAc also significantly inhibited PTL-induced apoptosis in MPO-high NB4 cells, whereas these MPO inhibitors did not affect PTL-induced apoptosis in MPO-low U937 cells. MPO silencing with MPO-specific siRNA transfection...
significantly decreased the level of PTL-induced apoptosis only in MPO-high NB4 cells in a dose-dependent manner. Additionally, an induced overexpression of wild-type MPO gene in K562 cells led to a significant increase in the PTL-induced ROS generation, glutathione depletion, activation of caspase cascades, and mitochondrial pathway-mediated cell death, as compared to parental K562 cells. These findings clearly indicate that PTL-induced ROS generation and apoptosis are critically associated with MPO expression in leukemia cells.

The specific ROS responsible for PTL-induced apoptosis in MPO-high leukemia cells was not investigated in this study. MPO is an endogenous lysosomal enzyme that removes H$_2$O$_2$ and catalyzes the formation of toxic hypochlorous acid (HOCl) (Hampton et al., 1998; Klebanoff, 2005; Sawayama et al., 2008). HOCl interacts with other small molecules to produce various ROS including hydroxyl radicals (-OH), singlet oxygen ($^{1}$O$_{2}$), peroxynitrite (ONOO$^{-}$), and ozone (O$_3$) (Hampton et al., 1998). Previous studies have demonstrated that MPO-derived chlorinated oxidants and -OH play key roles in inducing oxidative stress-mediated apoptosis in myeloid leukemia cells that are treated with EGCG (Nakazato et al., 2007), with ROS being responsible for triggering apoptosis. It was suggested that H$_2$O$_2$ produced in the presence of PTL is converted to HOCl by MPO. However, HOCl was not found to be responsible for directly inducing apoptosis as MPO-induced apoptosis was blocked by an O$_2$ scavenger and an -OH scavenger in MPO-positive leukemia cells (Nakazato et al., 2007). It can be suggested that a reaction between HOCl with O$_2$ may result in the formation of a hydroxyradical that could directly induce apoptosis of MPO-positive leukemia cells.

NF-κB is a transcription factor that plays a key role in regulating cell proliferation, apoptosis, stress responses, and cell signaling pathways (Hayden and Ghosh, 2008; Baud and Karin, 2009). It was shown that PTL potentially inhibits NF-κB activity by inhibiting IκB kinase complex, resulting in sustained cytoplasmic retention of NF-κB (Bork et al., 1997; Suvannasankha et al., 2008). Since NF-κB is aberrantly activated in the CD34+CD38- LSC population and is associated with resistance to chemotherapy in AML (Guzman et al., 2001), it is possible that PTL could be used as an agent to preferentially eradicate LSC. However, we found that PTL inhibits NF-κB only in the MPO-high leukemia cells. Likewise, PTL-induced downregulation of a variety of NF-κB-regulated
antiapoptotic molecules, such as Bcl-xL, Mcl-1, the X-linked inhibitor of apoptosis protein (XIAP), and survivin (Khoshnan et al., 2000; Taguchi et al., 2006) were documented only in the MPO-high leukemia cells. PTL-induced truncation of the BH3-only protein Bid (tBid), and induction of the proapoptotic targets PUMA and Bak as well as phosphorylation of c-Jun N-terminal kinase phosphorylation (p-JNK) were also observed selectively in the MPO-high leukemia cells.

LSC usually exists in a quiescent state and is therefore, unlikely to respond to standard anti-leukemia chemotherapeutic agents that preferentially eradicate actively cycling cells (Holyoake et al., 1999; Graham et al., 2002; Guan et al., 2003; Guzman et al., 2005). The persistence of LSC following chemotherapy may be a major factor contributing to relapse (Jordan and Guzman, 2004; van Rhenen et al., 2005). For these reasons, development of novel therapeutic agents that specifically target the LSC population without affecting normal hematopoietic stem cells is therefore crucial (Guzman et al., 2005). Since NF-κB is constitutively activated in the LSC population (Guzman et al., 2001), PTL can be considered a LSC-specific therapeutic agent. These primitive AML cells may be more sensitive to changes in oxidative stress than normal hematopoietic stem cells, and the resultant increase in ROS may contribute to AML-specific cell death (Guzman et al., 2005). As MPO was considered a crucial determinant of PTL-induced apoptosis in leukemia cells, we evaluated MPO expression in the primary AML cells with respect to CD34 and CD38 antigen coexpression. MPO was highly expressed in the CD34+CD38- leukemia cells obtained from the MPO-high AML cases. In contrast, MPO expression was very low in the CD34+CD38- leukemia cells obtained from MPO-low AML cases. PTL-induced apoptosis of CD34+CD38- LSC candidates was significantly higher in MPO-high AML compared to MPO-low AML cases. This finding suggests that PTL has a selective effect on MPO-high CD34+CD38- LSC. PTL-induced apoptosis was negligible in the CD34+ normal bone marrow cells, in which MPO expression was very low.

Taken together, MPO molecule is demonstrated to be functionally related to PTL-induced apoptosis, ROS generation, GSH depletion and the down-regulation of various anti-apoptotic molecules such as NF-κB in leukemia cells and CD34+CD38- LSC candidates. Therefore, the level of MPO expression within AML cells may be a potential marker for predicting the sensitivity of
leukemia cells and LSC to PTL-induced cell death. Our findings indicate that PTL treatment can potentially be considered as a promising targeted therapy for leukemia bulk and LSC in MPO-high AML cells. In addition, as conventional chemotherapeutic agents may induce NF-κB activation in leukemia cells, PTL can be combined with several common anti-leukemia drugs to overcome chemoresistance and enhance therapeutic responses.
References


Nakazato T, Sagawa M, Yamato K, Xian M, Yamamoto T, Suematsu M, Ikeda Y, and Kizaki M


Footnotes

This work was supported by the National Research Foundation Grant funded by the Korean Government [2009-0074042] and the Korean Government (MEST) [2010-0028371].
Legends for figures

Fig.1 Differential sensitivity of leukemia cell lines to PTL-induced apoptosis according to MPO expression. (A) Constitutive expression of MPO protein in leukemia cell lines was examined by flow cytometry (upper) and western blot analysis (lower) using the appropriate antibodies. (B) MPO activity of the leukemia cell lines was measured using a human MPO enzyme immunometric assay as described in Materials and methods. (C) MPO-low cell lines (K562, U937, KG1, OCI-AML3) and MPO-high cell lines (HL60, NB4, Kasumi-1, MOLM13) were exposed to the indicated concentration of parthenolide (PTL) for 24 hours, and then the percentage of apoptotic cells was determined by annexin-V/propidium iodide (PI) staining using flow cytometry (left). Cells were cultured with 10μM PTL for 24 hours, fixed with 70% Et-OH and stained with PI, after which the percentage of sub-G1 populations was determined by flow cytometry (right). (D) After treatment with 10μM PTL for 24 hours, the disruption of mitochondrial membrane potential (MMP) was measured by DiOC6 incubation and flow cytometry analysis in U937 and NB4 cells as described in Materials and methods (upper). Cytoplasmic translocation of cytochrome-C and Smac/DIABLO protein was evaluated by western blot analysis of the cytoplasmic and mitochondrial fraction (lower). (E) Cleavage of caspase-8, -9, -3 and PARP was examined in U937 and HL60 cells 24 hours after PTL treatment using western blot analysis (upper). The cells were exposed to 10μM PTL for 24 hours in the presence or absence of caspase inhibitor, z-VAD-fmk (20μM), after which the percentage of Annexin V-positive apoptotic cells was determined by flow cytometry (lower). Note that α-tubulin served as a loading control. Columns, mean value of three independent experiments; bars, SD. Cyto, cytoplasmic fraction; Mito, mitochondrial fraction. *P < 0.05, **P < 0.01, ***P < 0.001; NS, not significant

Fig.2. PTL-induced ROS generation with depletion of GSH in relation to MPO expression. (A) MPO-low cell lines (K562, U937) and MPO-high cell lines (HL60, NB4, Kasumi-1) were treated with 10μM PTL for 5 hours in the presence or absence of prior treatment with 1mM of antioxidant, N-acetylcysteine (NAC), for 1 hour. After incubation, intracellular reactive oxygen species (ROS)
generation was measured using flow cytometric analysis as described in Materials and methods. The representative histograms (left) and bar graphs (right) show a greater extent of ROS generation in MPO-high leukemia cell lines. Cells were treated with 10μM PTL for 24 hour with or without NAC pretreatment, and then the fraction of apoptotic cells (B) and the intracellular glutathione (GSH) depletion (C) was evaluated using flow cytometry and luminescence spectrometer, respectively. (D) U937 and NB4 cells were treated with PTL for 24 hour in the absence or presence of ABAH (100μM, 24 hours), or ScAc (0.5mM, 3 hours) pretreatment, and then the percentage of apoptotic cells was determined by flow cytometric analysis (left and right). (E) U937 and NB4 cells were transfected with specific-MPO siRNA as described in Materials and methods. Mock siRNA trasfection was conducted as control experiment. After MPO gene silencing for 24 hours, total cell lysates of U937 and NB4 cells were analyzed by western blotting using antibody against MPO. MPO/α-tubulin ratio according to amount of MPO siRNA was shown below the blots (upper). The cells were treated respectively with PTL for 24 hours in the absence or presence of specific-MPO siRNA, and then the percentage of apoptotic cells was determined by flow cytometric analysis (lower). Columns, mean of three independent experiments; Bars, SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig.3 Alterations in the level of anti- or pro-apoptotic molecules with PTL treatment. (A and B upper) MPO-low U937 and MPO-high HL60 cells were treated with 10μM PTL for 24 hours, after which western blot analysis was conducted using the appropriate antibodies. (B lower) Cells were exposed to PTL for 24 hours with or without prior incubation with JNK inhibitor SP600125 or p38 inhibitor SB203580 for 3 hours, and then the percentage of apoptotic cells was determined by flow cytometry. Columns, mean of three independent experiments; Bars, SD.

Fig.4. Induced overexpression of MPO enhanced sensitivity to PTL-induced apoptosis. K562 cells were transfected with wild-type MPO (K562/MPO), mutant MPO (K562/R569W), and pCI empty control (K562/Control) cDNA construct using Nucleofection as described in Materials and methods. After electroporation, stable transfectant clones were selected and used for further
experiments. (A) Heavy (α) subunit and precursor MPO protein expression in each transfected cell lines were evaluated using flow cytometry (left) and western blot analysis (right). (b1) Each transfectant was exposed to the indicated concentration of PTL for 24 hours, and then the percentage of apoptotic cells was determined by flow cytometry. The histograms (b2, left) and bar graphs (b2, right) show the percentage of sub-G1 populations measured by flow cytometry in K562 and K562/MPO cells 24hrs after treatment with 10μM PTL. The disruption of mitochondrial membrane potential (ΔΨm) was measured by DiOC6 incubation and flow cytometry analysis (b3, left), and the cytoplasmic translocation of cytochrome-C and Smac/DIABLO protein examined by western blot analysis (b3, right). The cleavage of caspase-8, -9, -3 and PARP was examined in two cell lines 24 hours after PTL treatment using western blot analysis (b4, left). K562 and K562/MPO cells were exposed to 10μM PTL for 24 hours in the presence or absence of the caspase inhibitor z-VAD-fmk, after which the percentage of apoptotic cells was determined by flow cytometry (b4, right). K562 and K562/MPO cells were cultured with 10μM PTL for 5 hours in the presence or absence of prior incubation with 1mM NAC, after which intracellular ROS generation (C, upper and lower) and GSH depletion (D) was measured using flow cytometry. (E) K562 and K562/MPO cells were treated with 10μM PTL for 24 hr, and then western blot analysis was conducted using the appropriate antibodies. Columns, mean of three independent experiments; Bars, SD. *P < 0.05, **P < 0.01.

Fig.5. Differential sensitivity of primary AML cells to PTL-induced apoptosis according to MPO expression. (A) Constitutive MPO activity of primary leukemia cells obtained from MPO-high(hi) AML (n=6), MPO-low(lo) AML (n=4) cases as well as CD34-positive normal bone marrow cells (n=4) was measured using human MPO enzyme immunometric assay. Primary MPO-high and MPO-low AML cases were arbitrarily defined according to the fraction of leukemic blasts expressing MPO as described in Materials and methods. Primary MPO-high AML cells, MPO-low AML cells, and CD34-positive normal bone marrow cells were treated with 10μM PTL for 24 hr, then (B) the fraction of apoptotic cells and (C) intracellular ROS generation were evaluated by flow cytometry (upper and lower), and (D) GSH depletion was examined using luminescence spectrometer. Columns,
mean of three independent experiments; Bars, SD.

**Fig.6. Differential sensitivity of CD34+CD38- leukemia stem cells to PTL-induced apoptosis according to MPO expression.** (A) Representative flow cytometric dot plot analysis of CD34, CD38, and MPO expression in primary leukemia cells obtained from a MPO-low (left upper), a MPO-high AML (left middle) and normal bone marrow CD34+ case (left lower). Histograms in the inner box showed the proportion of MPO-positive cells in the total leukemia cells in each case. MPO expression in the gated CD34+CD38- leukemia stem cells (LSC) fraction was determined in the MPO-low (right upper) and a MPO-high AML case (right middle) using three-color flow cytometric analysis. Fraction of MPO-positive cells was significantly higher in the MPO-high AML (n=10) cases compared to MPO-low AML (n=4) cases and normal bone marrow CD34+ cells (n=3) (right lower). (B) Cells from MPO-high (n=10), MPO-low (n=4) as well as normal CD34-positive bone marrow cells (n=3) were treated with 10μM PTL for 24 hours, and then the proportion of apoptotic cells in the CD34+CD38- LSC fraction and normal CD34+ bone marrow cells was determined by flow cytometry as described in materials and methods. (C) Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were irradiated and transplanted with primary human leukemia cells obtained from MPO-high AML and MPO-low AML cases, or with CD34-positive bone marrow cells harvested from healthy donors. Before transplantation, the cells were treated with 10μM PTL or DMSO control for 24 hours. After 6 to 8 weeks, animals were sacrificed and bone marrow was analyzed for the presence of human CD45-positive cells by flow cytometry. Each ■ or ▲ represents a single animal analyzed for level of human cell engraftment in the bone marrow. Mean engraftment is indicated by the horizontal bars.
Table 1. PTL-induced apoptosis of primary AML cells and normal CD34-positive bone marrow cells according to MPO expression

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex/Age</th>
<th>FAB classification</th>
<th>MPO (%)*</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control**</td>
</tr>
</tbody>
</table>

**AML cases**

1. F/39 M1 18.4 0.3 3.5
2. M/39 M4 9.7 0.4 1.1
3. M/31 M2 4.1 0.8 0.1
4. M/69 M2 6.6 0.7 1.0
5. M/28 M2 48.2 0.6 44.2
6. F/33 M0 59.9 0.7 45.2
7. F/59 M1 66.0 0.1 45.6
8. M/65 M1 46.0 0.7 42.3
9. M/53 M4 46.7 0.8 43.0
10. F/25 M4 70.5 0.1 46.6

**Healthy donors**

11. M/46 3.7 2.1 9.3
12. M/53 8.8 5.6 7.1
13. F/49 1.0 3.4 8.9
14. F/54 0.1 3.4 9.5

*MPO(%), percentage of MPO-positive cells in the total mononuclear cells

**After treatment of primary AML cells or normal CD34-positive bone marrow cells with 10μM of PTL for 24 hours, the proportion of apoptotic cells was evaluated using flow cytometry

***Control, DMSO
Fig 1.

A. MPO-low

- K562: 6.0%
- U937: 4.8%
- KG1: 4.7%
- OCI-AML3: 5.2%

MPO-high

- HL60: 98.8%
- NB4: 92.8%
- Kasumi-1: 99.7%
- MOLM13: 73.6%

B. MPO activity (ng/10^6 cells)

- K562
- U937
- HL60
- NB4
- Kasumi-1

(Im mature MPO)
Precursor MPO

Heavy(α)-subunit
MPO (Mature MPO)

α-tubulin
Fig 2.

A. 

ROs production

Counts

FL1-Height

ROS production

Counts

FL1-Height

B. 

Control

PTL (10μM, 5h)

NAC (1mM, 1h) + PTL

C. 

Control

PTL (10μM, 24h)

NAC (1mM, 1h) + PTL

Apoptosis (%)
Fig 2.

D.

U937

Control  PTL  PTL + ABAH  PTL + ScAc

NB4

PI

Annexin V-FITC

Apoptosis (%)

PTL  PTL + ABAH (100 μM)  PTL + ScAc (0.5 mM)

P < 0.001

E.

U937  NB4

Control  Mock  MPO siRNA (nM)  Control  Mock  MPO siRNA (nM)

Mock  50  100  200  400  Mock  50  100  200  400

↓ Heavy(α)-subunit MPO  ↓ α-tubulin

Control  PTL (10 μM)

Apoptosis (%)

Control  Mock  MPO siRNA

Mock  50  100  200  400 (nM)  Mock  50  100  200  400 (nM)
Fig 3.

A.

<table>
<thead>
<tr>
<th></th>
<th>U937</th>
<th>HL60</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- NF-kB (p65)
- Bcl-xL
- Mcl-1
- XIAP
- Survivin
- BID
- tBID
- Bak
- PUMA
- α-Tubulin

B.

<table>
<thead>
<tr>
<th></th>
<th>U937</th>
<th>HL60</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- p-JNK
- JNK
- p-p38
- p38

Apoptosis (%)

- Control
- PTL
- PTL + SP600125 (10μM)
- PTL + SB203580 (10μM)

U937: H937
HL60: HL60
Fig 4.

A. 

A1) 

B. 

B1) 

B2) 

B3) 

B4)
**Supplementary data.** PTL-induced apoptosis in CD34+CD38- cells and normal CD34+ cells according to MPO expression

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex/Age</th>
<th>FAB classification</th>
<th>LSC (%) (CD34+CD38-)</th>
<th>MPO (%) in LSC*</th>
<th>Apoptosis (%) Control</th>
<th>PTL(10μM)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>F/63</td>
<td>M4</td>
<td>33.3</td>
<td>6.7</td>
<td>43.3</td>
<td>43.3</td>
</tr>
<tr>
<td>16</td>
<td>M/26</td>
<td>M2</td>
<td>26.4</td>
<td>5.3</td>
<td>25.0</td>
<td>33.3</td>
</tr>
<tr>
<td>17</td>
<td>M/64</td>
<td>M5</td>
<td>4.3</td>
<td>1.1</td>
<td>21.6</td>
<td>21.1</td>
</tr>
<tr>
<td>18</td>
<td>M/31</td>
<td>M2</td>
<td>0.5</td>
<td>2.0</td>
<td>23.6</td>
<td>25.1</td>
</tr>
<tr>
<td>19</td>
<td>M/35</td>
<td>M2</td>
<td>0.8</td>
<td>81.1</td>
<td>5.9</td>
<td>87.8</td>
</tr>
<tr>
<td>20</td>
<td>F/56</td>
<td>M4</td>
<td>2.0</td>
<td>96.0</td>
<td>51.4</td>
<td>84.1</td>
</tr>
<tr>
<td>21</td>
<td>M/66</td>
<td>M4</td>
<td>11.7</td>
<td>9.04</td>
<td>27.8</td>
<td>100.0</td>
</tr>
<tr>
<td>22</td>
<td>F/29</td>
<td>M2</td>
<td>2.7</td>
<td>59.1</td>
<td>3.5</td>
<td>80.0</td>
</tr>
<tr>
<td>23</td>
<td>F/28</td>
<td>M2</td>
<td>1.3</td>
<td>90.6</td>
<td>37.3</td>
<td>71.6</td>
</tr>
<tr>
<td>24</td>
<td>F/48</td>
<td>M1</td>
<td>1.0</td>
<td>98.5</td>
<td>12.5</td>
<td>93.8</td>
</tr>
<tr>
<td>25</td>
<td>M/19</td>
<td>M2</td>
<td>5.8</td>
<td>96.5</td>
<td>3.9</td>
<td>96.6</td>
</tr>
<tr>
<td>8</td>
<td>M/65</td>
<td>M1</td>
<td>3.2</td>
<td>76.4</td>
<td>4.2</td>
<td>91.3</td>
</tr>
<tr>
<td>9</td>
<td>M/53</td>
<td>M4</td>
<td>2.4</td>
<td>94.0</td>
<td>8.0</td>
<td>90.0</td>
</tr>
<tr>
<td>10</td>
<td>F/25</td>
<td>M4</td>
<td>27.2</td>
<td>75.9</td>
<td>0.0</td>
<td>78.8</td>
</tr>
</tbody>
</table>

*MPO(%), percentage of MPO-positive cells in the CD34+CD38- bone marrow cells

**After treatment of CD34+CD38- bone marrow cells with 10μM of PTL for 24 hours, the proportion of apoptotic cells was evaluated using flow cytometry

***Control, DMSO

Abbreviations: LSC, leukemia stem cells