TPEN selectively eliminates lymphoblastic B cells from pediatric acute lymphoblastic leukemia patients

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Abstract: B-acute lymphoblastic leukemia (B-ALL) is a hematologic disorder characterized by abnormal proliferation and accumulation of immature B-lymphoblast arrested at various differentiation stages. Despite some advances in treatment, there is still an important percentage of pediatric patients with precursor-B ALL who relapsed. Therefore, alternative therapies are needed to improve cure rates for pediatric patients. TPEN is a pro-oxidant agent capable of selectively inducing apoptosis in leukemia cells. Consequently, TPEN has been suggested as a potential agent for oxidative therapy. However, it is not yet known whether TPEN can selectively destroy leukemia cells in a more disease-like milieu e.g., bloodstream and bone marrow (BM) in vivo. In this investigation, we report for the first time that TPEN significantly induces apoptosis in CD34+/CD19+ cells from whole bone marrow de novo B-ALL (n=5) and refractory B-ALL (n=6) patients by oxidative stress (OS, n=8). We found that TPEN significantly increased not only positive cell counts for the oxidation of the stress sensor protein DJ-1 as a sign of the formation of H2O2, but also significantly increased positive cell counts for the proapoptotic protein TP53, PUMA, and CASPASE-3 as indicative of apoptosis in B-ALL cells irrespective of diagnostic status (de novo or refractory) and sex. Understanding the TPEN-induced cell death in leukemia cells provides insight into more effective therapeutic prooxidant-inducing anticancer agents.

Keywords: Acute leukemia; CASPASE-3; chemoresistant; DJ-1; TP53; PUMA; reactive oxygen species; signaling; TPEN

1. Introduction

B-acute lymphoblastic leukemia (B-ALL) is a neoplasm of immature B-cell precursors characterized by abnormal proliferation and accumulation of immature lymphoblast arrested at pre-pro-B cell or pro-B cell stages [1]. B-ALL typically affects children younger than 6 years but also in older children and in adult populations [2,3]. The diagnosis is established by immunophenotyping, commonly by flow cytometry, which shows immature B lineage. Many cases of B-ALL harbor recurrent chromosomal abnormalities, which are critical determinants of prognosis [4]. Despite intensive classical chemotherapy and other available targeted therapies [5], about 20% of children with precursor-B ALL will relapse (e.g., [6]). Therefore, alternative therapies are needed to improve cure rates for pediatric patients with B-cell relapsed leukemia.

Oxidation therapy offers an important therapeutic opportunity to eliminate B-ALL [7-9]. The administration of exogenous agents aimed at selective production of reactive oxygen species (ROS) can impair the redox balance of leukemia cells [10,11] leading to

apoptosis -a regulated cell death process [12], which is commonly altered in those cells [13-15]. Accordingly, several ROS-producing agents have demonstrated selectively enhancing the cancer ROS levels and apoptosis [16,17], including the lipid-soluble zinc metal chelator TPEN (N, N, N', N'-Tetrakis (2-pyridylmethyl) -ethylenediamine).   Remarkably, TPEN induces apoptosis in several cancer cell lines [18-23], including leukemia cells [24] through the generation of high ROS levels and oxidative stress   (OS, [25-27]). Mechanistically, it has been shown that TPEN induced apoptosis through 2 independent but complementary pathways triggered by hydrogen peroxide (H2O2). In fact, TPEN/ (H2O2) activates not only the transcription factor NF-κB, TP53, c-JUN; up-regulated proapoptotic proteins such as BAX/PUMA but also induces the loss of mitochondrial membrane potential (ΔΨm), activation of protease CASPASE-3 and NADH-dependent oxidoreductase AIF, all leading to nuclear chromatin condensation and DNA fragmentation, typical of apoptosis [25-27]. Although the cytotoxic effect of TPEN on leukemia cells has been tested either in model cells (e.g., acute lymphoblastic leukemia Jurkat cells [25]; chronic myeloid leukemia K562 cells [26]; acute promyelocytic NB4 cells [24]), or in ex vivo/isolated and purified cells from leukemia bone marrow samples [27], no data are available to establish whether TPEN induces apoptosis in B-cells from ex vivo whole bone marrow (WBM) pediatric patients with refractory B-ALL.

To gain insight into this issue, we wanted to determine whether TPEN treatment induces apoptosis in B-ALL cells in WBM from refractory patients. To this aim, we evaluated the effect of TPEN on CD34+/ CD19+ leukemic cells (de novo B-ALL n=5, refractory B-ALL n=6) with selected OS markers (e.g., oxidation of D-1 protein, oxDJ-1), and cell death (e.g., BH3-only protein PUMA, transcription factor TP53, execution protein CASP3) by using flow cytometry analysis.   We found that TPEN induces apoptosis in B-ALL cells via the generation of H2O2, switch on of TP53 and PUMA, activation of CASP-3, and switch off of antioxidant protein DJ-1. Taken together these observations suggest that TPEN efficiently eliminates B-ALL cells in pediatric patients with de novo and/or refractory B-ALL.

2. Results

2.1.   TPEN induces a reduction of CD34+/CD19+ population, and activation of CASPASE-3 in lymphoblastic B-cells derived from bone marrow chemoresistant leukemia pediatric patients

As a first approached, we evaluated whether TPEN induces apoptosis in de novo (CD34+/ CD19+) B-ALL cells from whole bone marrow (WBM) pediatric patients (n=3; code #72750; #19105; #62232; Table 1, raw 1–3). The WBM samples were left untreated or exposed to TPEN increasing concentrations (50, 100, 200, and 500 ⊚M) for 24 h. As shown in Fig. 1A, TPEN significantly reduced the CD34+/ CD19+ population in a concentration-independent manner e.g., 93±3.4% (untreated) vs. 86.3±10.7% (50), 71±7.2% (100), 66.3±5.7% (200), and 62.3±8% (500 ⊚M TPEN) according to double (CD34+/ CD19+) flow cytometry analysis. Further analysis (red broken lines in Fig. 1B) showed that TPEN at 50, 100, 200, and 500 (⊚M) increased the percentage of CASP-3 activation by ~57%, ~86%, ~86% and ~88%, respectively. Because TPEN (100 ⊚M) was the minimal concentration at which CASP-3+ was activated as indicative marker of apoptosis cell death (Fig. 1C, i.e., ~7-fold increase CASP-3 activation in CD34+/ CD19+ B-cells, n=3), this concentration was selected for further experimental procedures.

Table 1. Characteristic profile of cells from de novo (code number in blue) and refractory (code number in red) B-ALL pediatric patients.

#	Code	Diag.	Diagnostic Status	WBC	Blasts (%)	Sex	Age (years)	Kariotype	Immunophenotype
1	72750	B-ALL	*de novo*	139,300	85%	M	13	Complex Karyotype	97% B cells: CD45w, CD19+, CD79a+, CD22+, CD20+/-, CD24+, CD9+, CD10+, CD34+/-, CD38+, TdT+, CD123w, CD66+/-, cIgM+. cMPO-, CD13-, CD33-, CD117-, cCD3-, sIgM-
2	19105	B-ALL	*de novo*	10,500	50%	F	12	Complex Karyotype	95% B cells: CD45w/-, CD34 +/-, CD19+, CD79a+, CD20+/-, CD10+, CD38+, Tdt+, CD22+, CD24+, CD95+
3	62232	B-ALL	*de novo*	81,300	92%	M	8	Complex Karyotype	92% B cells: CD45w, CD19+, CD79a+, CD22+, TdT+, CD10+, CD20+/-, CD24+, CD9+
4	32765	B-ALL	refractory	166,620	90%	F	13	t(9:22)-, MLL+	97% B cells: CD45w, CD19+, CD79A+, CD22+, TdT het, CD34 het, CD38+, CD15+, NG2CD24 het, CD9+ y CD123+. CD3-, CD7-, MPO-, CD10-, cIgU-, sIgM-, CD66C-, CD21-, CD13-, CD33-, CD20-. CD15 and NG2 + associated to 11q2.3.
5	48983	B-ALL	refractory	23,300	50%	F	9	Complex Karyotype hyperdiploid mosaicism, T12:21+	84% B cells: CD45w, CD34+, CD19+, CD79a+, CD22+, CD10+, CD20 het, TdT+, CD9+, CD24+, CD123+, CD66c+. Neg. for CD3, MPO, CD7, CD13, CD33, IgM, cIgU, CD21.
6	06600	B-ALL	refractory	2,540	61%	M	16	NP	61% B cells CD45w, CD34+, CD19+, CD20 het, CD10+, CD38+, CD66c+ y CD123+. CD15-, NG2-, CD21-
7	79426	B-ALL	refractory	4,700	16%	F	5	51-54, XX, +del(6)(q23), +8, +14, +14, +21, +22[cp7]/46, XX[20]	84% B cells: CD19+, CD34 het., CD10+, CD38+, CD66c+, CD123+. CD45-, CD20-, CD15-, NG2-
8	81571	B-ALL	refractory	3,500	12%	M	3.5	44-45, XY, dic(1;12)(q21;p13), t(5;17)(q35;q21), der(7;12)(q10;10), i(12)(p10)[cp5]/46, XY[5]	96% B cells: CD19+, CD34 partial, CD10+, CD38+, CD66c+, CD123 partial, CD15 partial. CD45-, CD20-, NG2-
9	11630	B-ALL	*de novo*	27,900	85%	F	7	46, XX, del(6)(q21)[6]/46, XX[15]	89% B cells: CD45w, CD19+, CD79a+, CD34+, CD22+, TdT+, CD20 het, CD10+, CD38+, CD123+, CD13 partial, CD33 partial, CD24+. cIgU-, sIgM-, MPO-, CD7-, CD3-, CD66c-, CD21- NG2-, CD15-.
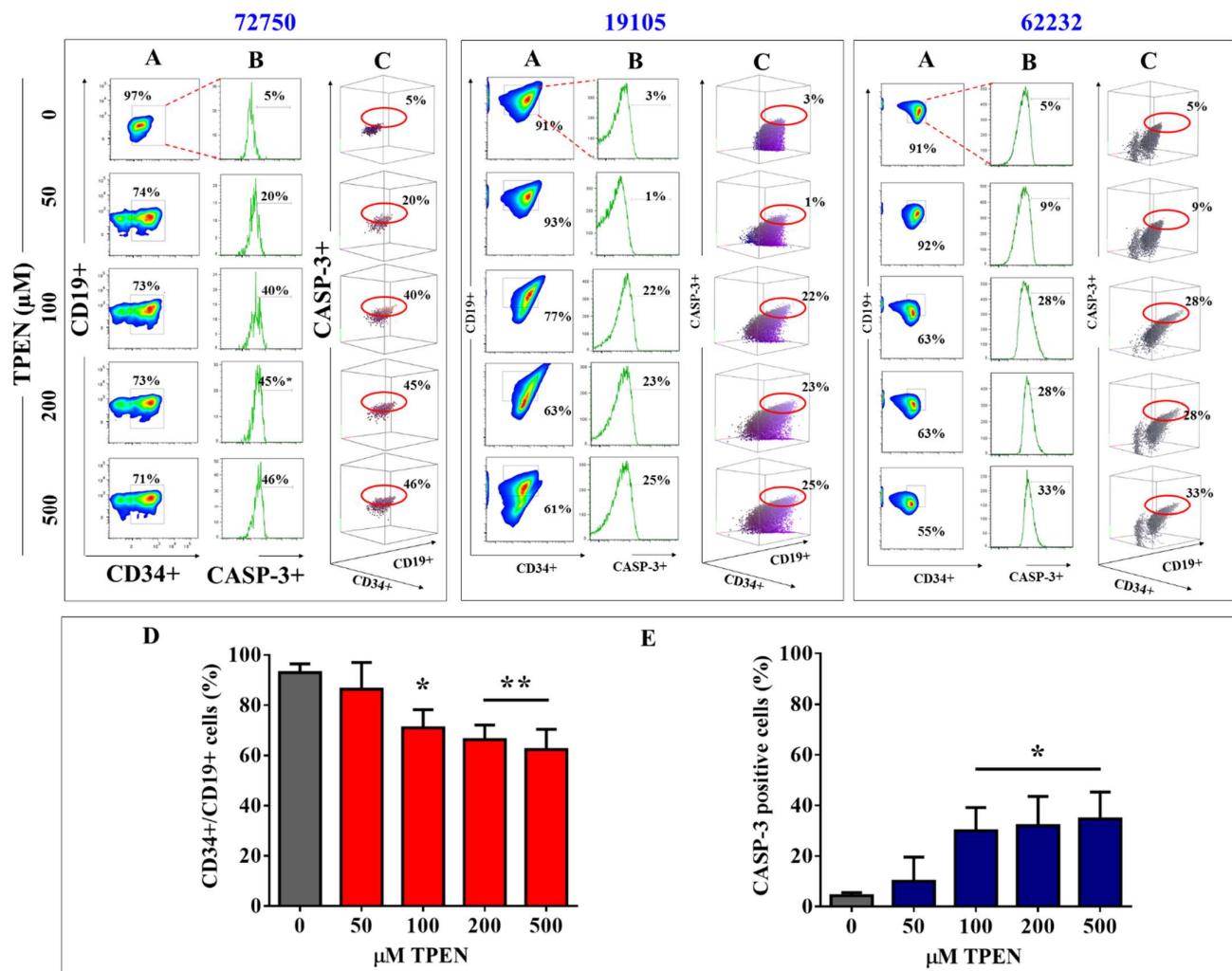
Figure 1. TPEN induces reduction of CD34+/CD19+ cell population and activation of CASPASE-3 in cells from de novo (code number in blue) B-ALL patients.

(A) Representative 2D density plot showing CD34 (x axis) and CD19 (y axis) flow cytometry performed to leukemic cells (n=3) from whole bone marrow (WBM) de novo B-ALL patients treated with TPEN (0, 50, 100, 200, and 500 μM) at 37 °C for 24 h; (B) Representative histograms showing CASPASE-3-positive subset from CD34+/CD19+ cells. (C) Representative 3D density plot showing CD34 (x axis), CD19 (z axis) and CASPASE-3 (y axis) positive cells. (D) and (E) represent the quantitative analysis of the parameters. *p<0.05; **p<0.01; ***p<0.001.

To validate the effect of TPEN on B-cells, we performed flow cytometry double analysis of CD19+/ CD34+ population in de novo (n=2) and refractory (n=6) B-ALL patients (Table 1, Table 1, raw 4–11). TPEN reduced the B-ALL blast population in all specimens (Fig. 2A). While the mean value of blast percentage in untreated cells was 51.4±8.0%, TPEN (100 ⊙M) significantly reduced the blast population to 31.9±10.4% (i.e., ~37% reduction, Fig. 2B). Likewise, flow cytometry 3D density plot analysis (CD19+/ CD34+ /

CASP-3+) indicated that TPEN increased (~88%) CASP-3 activation in leukemic B cells (e.g., 32±15%) compared to untreated cells (4.25±1%, Fig. 3A and 3B).
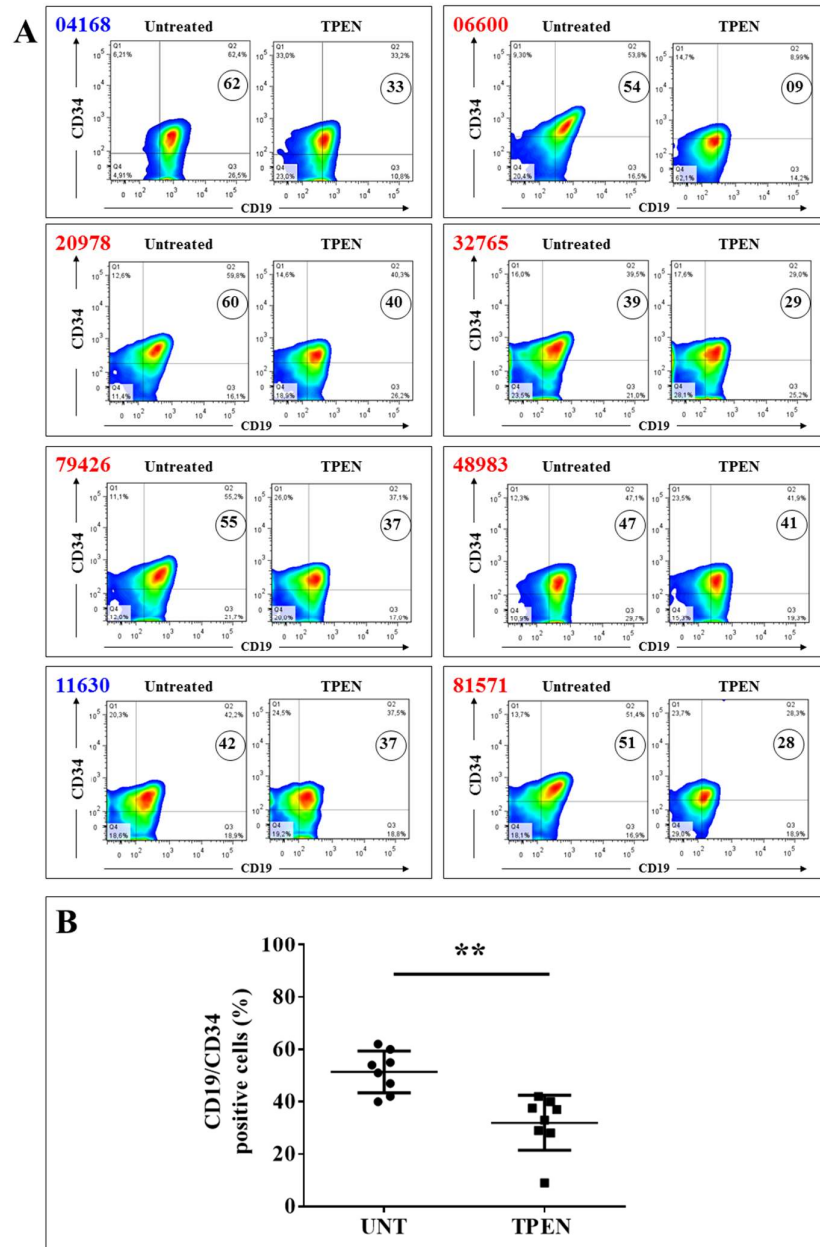
Figure 2. TPEN induces a reduction in the CD34+/CD19+ population in cells from de novo (code number in blue) and refractory (code number in red) B-ALL patients.

(A) Representative 2D density plot showing CD34 (y axis) and CD19 (x axis) flow cytometry double analyses (Q2) performed to leukemic cells (de novo (n=2) and refractory (n=6) B-ALL patients) from untreated or treated with TPEN (100 µM) at 37 °C for 24 h; (B) represents the quantitative analysis of the data from the quadrant Q2. *p<0.05; **p<0.01; ***p<0.001.
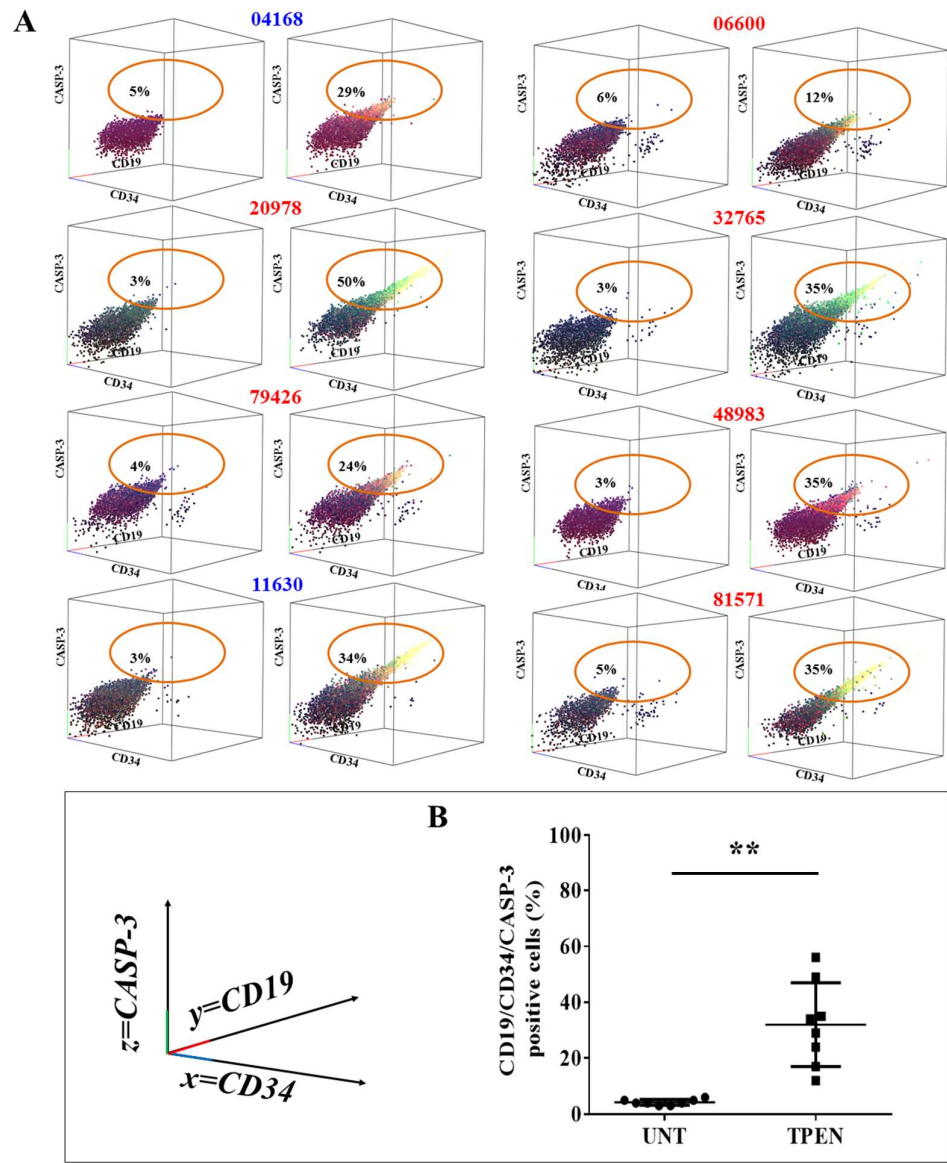
Figure 3. TPEN induces the activation of CASPASE-3 in the CD34+/CD19+ population in cells from de novo (code number in blue) and refractory (code number in red) B-ALL patients.

(A)   Representative 3D density plot showing the flow cytometry analysis of CD34 (x axis), CD19 (z axis) and CASPASE-3 (y axis) positive cells performed to leukemic cells (de novo (n=2) and refractory (n=6) B-ALL patients) from untreated or treated with TPEN (100 μM) at 37 °C for 24 h; (B) represents the quantitative analysis of the data. *p<0.05; **p<0.01; ***p<0.001.

3.2. TPEN simultaneously induces oxidation of DJ-1 and activation CASP-3 in cells derived from chemoresistant leukemia patients.

Next, we asked whether TPEN induces the oxidation of the stress sensor protein DJ-1 and activated CASP-3 in WBM cells. As shown in Fig. 4A, TPEN increased double staining of oxidized DJ-1+/CASP-3+ cells (e.g., 16.25±11%, n=8) compared to untreated cells (1.1±0.8%, n=8; Fig. 4B) by 94%.3.
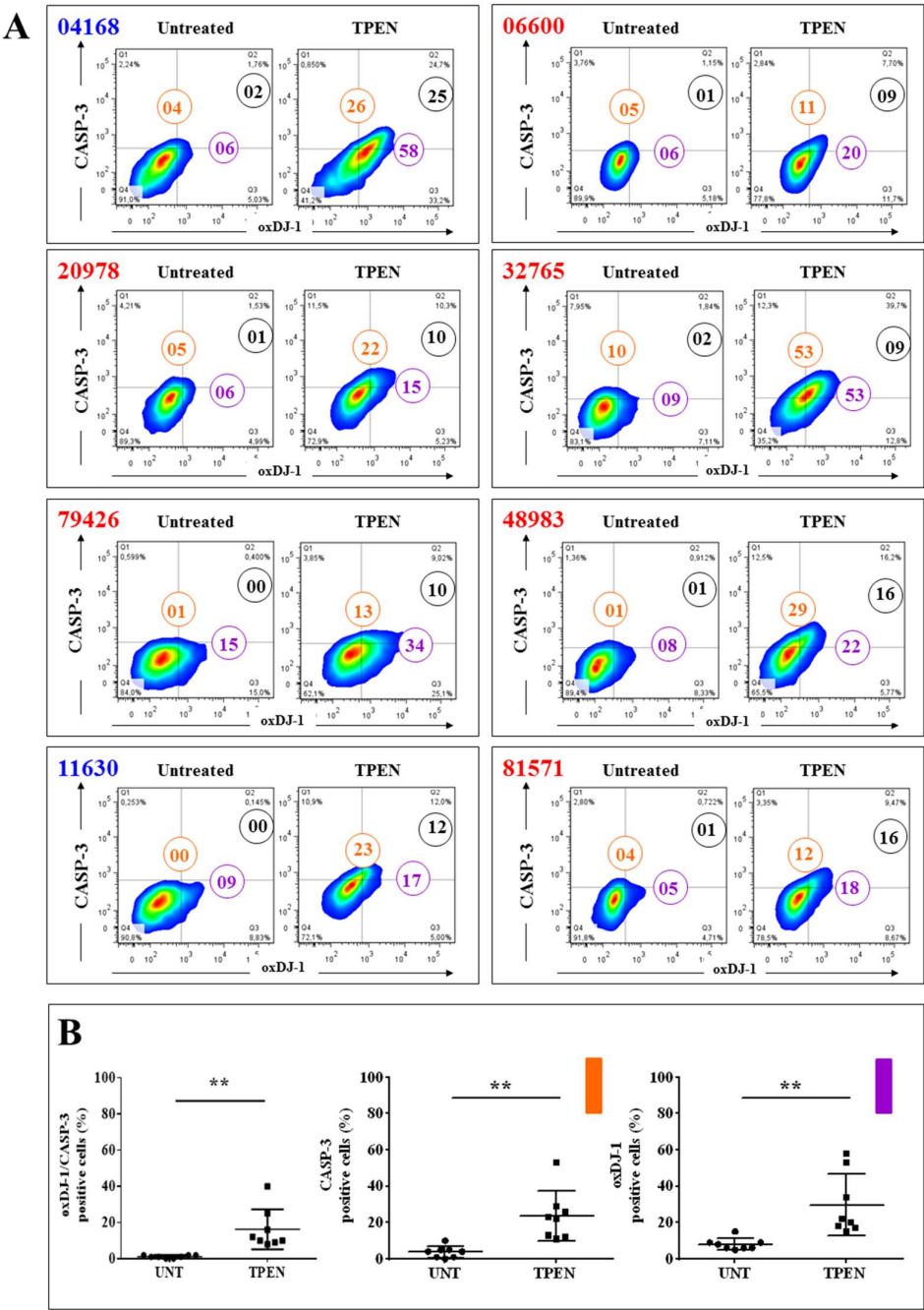
Figure 4. TPEN induces simultaneous activation of CASPASE-3 and oxidation of DJ-1 in cells from de novo (code number in blue) and refractory (code number in red) B-ALL patients.

(A)   Representative 2D density plot showing CASPASE-3 (y axis) and ox-DJ-1 (x axis) flow cytometry double analyses (Q2) performed to leukemic cells (de novo (n=2) and refractory (n=6) B-ALL patients) untreated or treated with 100 μM TPEN at 37 °C for 24 h; (B) represents the quantitative analysis of the data from the quadrant Q2. *p<0.05; **p<0.01; ***p<0.001.

3.3 TPEN up-regulates PUMA protein in cells derived from chemoresistant leukemia patients.

The above observations prompted us to evaluate whether TPEN was capable of inducing activation of protein PUMA -a death marker in chemoresistant B-ALL derived cells. Flow cytometry 3D density plot analysis (CD34+/CD19+/PUMA+) indicated that TPEN upregulated PUMA protein in leukemic B cells (27.5±9.1%, n=8) compared to untreated WBM cells (e.g., 5.25±2.7%, n=8; Fig. 5A and 5B) by 81%.
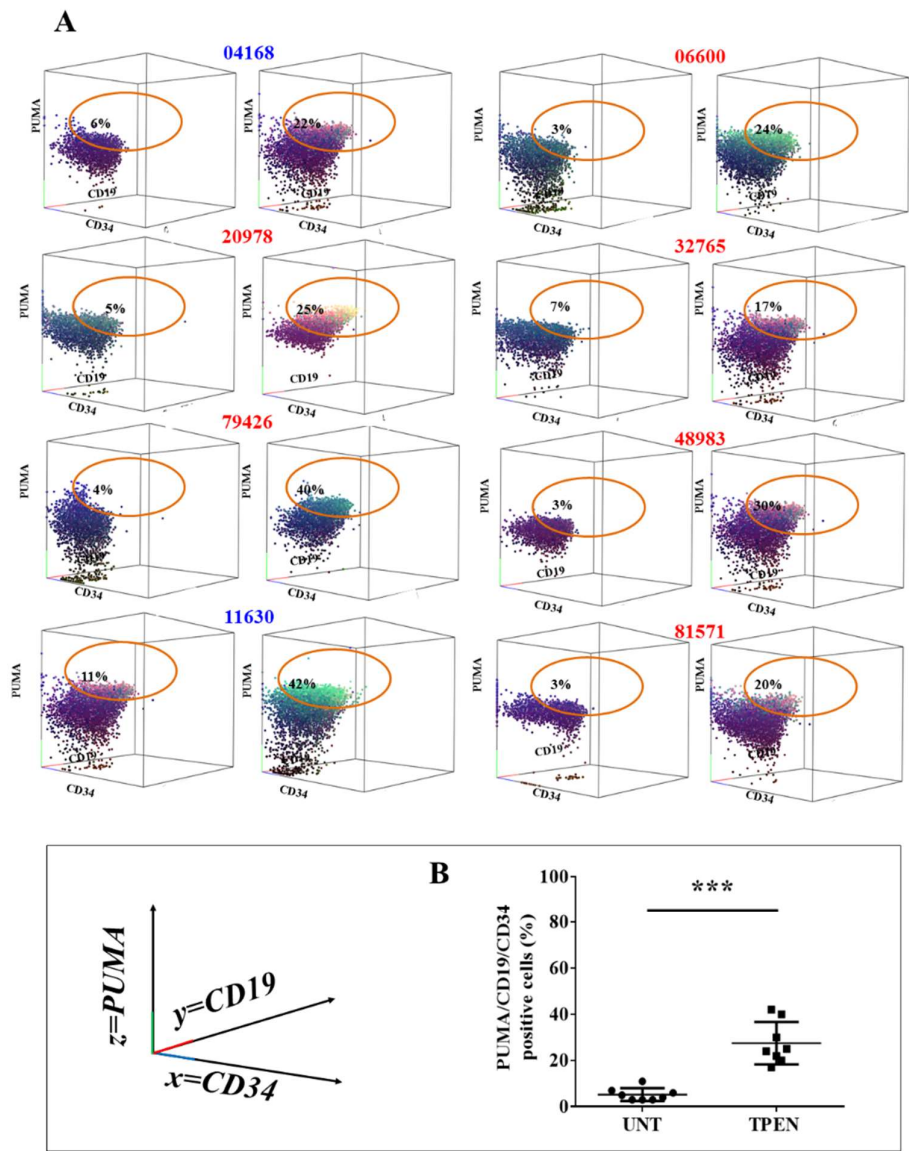
Figure 5. TPEN induces the activation of PUMA in cells from de novo (code number in blue) and refractory (code number in red) B-ALL patients.

(A) Representative 3D density plot showing the flow cytometry analysis of CD34 (x axis), CD19 (z axis) and PUMA (y axis) positive cells performed to leukemic cells (de novo (n=2) and refractory (n=6) B-ALL patients) untreated or treated with 100 μM TPEN at 37 °C for 24 h; (B) represents the quantitative analysis of the data. *p<0.05; **p<0.01; ***p<0.001.

3.4 TPEN simultaneously up-regulates PUMA protein and TTP53 transcription factors in cells derived from chemoresistant leukemia patients.

We then evaluated whether TPEN was capable of inducing activation of transcription factor TP53 concurrently with the pro-apoptotic protein PUMA in WBM samples from

chemoresistant B-leukemia patients. As shown in Fig. 6A, TPEN significantly increased double PUMA+/ TP53+ cells (e.g., 3.5±5.4% (Untreated) vs. 22 ±9.5%; Fig. 6B) by 67%.
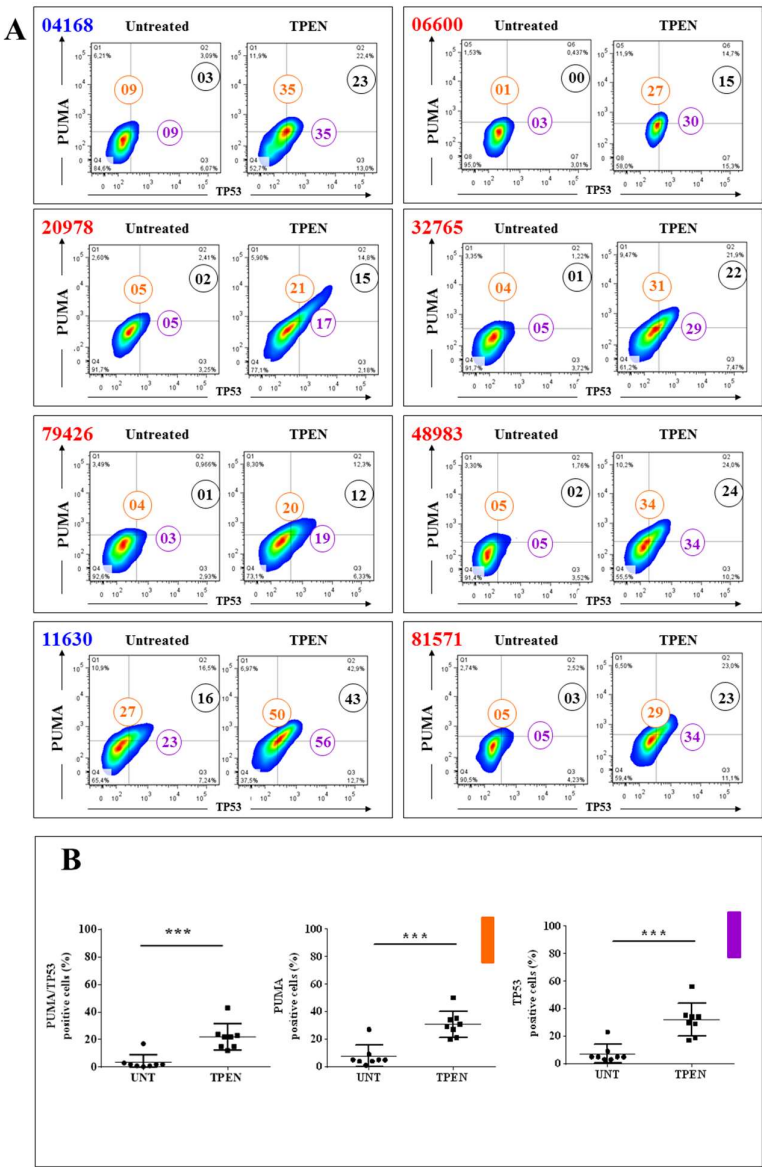
Figure 6

Figure 6. TPEN induces activation of TP53 and PUMA in cells from de novo (code number in blue) and refractory (code number in red) B-ALL patients.

 (A)   Representative 2D density plot showing PUMA (y axis) and TP53 (x axis) flow cytometry double analyses (Q2) performed to leukemic cells (de novo (n=2) and refractory (n=6) B-ALL patients) untreated or treated with 100 μM TPEN at 37 °C for 24 h; (B) represents the quantitative analysis of the data from the Q2 quadrant. *p<0.05; **p<0.01; ***p<0.001.

4. Discussion

Previous studies have shown TPEN as a pro-oxidant agent capable of inducing selective pro-apoptotic leukemia cell death through oxidative stress mechanism, involving generating H2O2, oxidation of stress sensor protein DJ-1, activation of transcription factor TP53 and pro-apoptotic effector BH-3 only protein PUMA, mitochondrial damage, activation of proapoptotic executor protein CASPASE-3, and disassembly of the nuclei in leukemia cell model (e.g., [25,26]) and in ex vivo isolated refractory leukemia cells from ALL bone marrow samples [27]. Consequently, TPEN has been suggested as a potential agent for oxidative therapy. However, it is not yet known whether TPEN can selectively destroy leukemia cells in a more disease-like milieu e.g., bloodstream and bone marrow (BM). BM is an important microenvironment in which deranged lymphocyte proliferation (e.g., B-ALL) occurs [28]. Since BM is routinely examined for diagnosis and disease prognosis, it represents an important source of biological material and clinical information. Here, we report for the first time that TPEN specifically induces apoptosis in de novo (n=5, i.e., patients with no clinical history of prior B-ALL disorder, or exposure to potentially leukemogenic therapies or agents) and refractory leukemia cells (n=6) from ex vivo whole bone marrow (n= 11) according to the detection of selected OS-signal and cell death markers in eight (n=8) CD34+/CD19+ (B-ALL) patients. We found that TPEN significantly increased not only the oxidation of stress sensor protein DJ-1 as indication of the formation of H2O2 and OS, but also increased the cell signal positive for the pro-apoptotic protein TP53, PUMA, and CASPASE-3 as indicative of apoptosis cell death. Therefore, in agreement with previous work [25,27], TPEN provokes apoptosis in lymphoblastic cells, e.g., in T-ALL and B-ALL cells through activation of intrinsic apoptotic pathway [12]. This investigation, however, differs from previous ones. First, TPEN induces apoptosis in ex vivo WBM B-ALL cells in a dose-independent manner. Indeed, the effective dose (ED50%) TPEN on ex vivo B-ALL cells was between 100 ⊚M-500 ⊚M whereas in vitro the ED50% TPEN on leukemia cells and ex vivo isolated T-ALL/B-ALL cells was 5 ⊚M [25,27]. Indeed, the TPEN concentration should rise in about 20-to 100-fold to be effective in ex vivo. Clearly, the toxicity of TPEN on cells is lessened by BM milieu. Therefore, although TPEN induces apoptosis in leukemia cells by OS-mechanism, the cytotoxic concentrations of TPEN vary hugely when applied to isolated versus non-isolated cell conditions. This observation should be taken into account when data migrate from preclinical experimental procedures to clinical trials. Interestingly, TPEN has been reported to be harmless to mice (e.g., at 5 mg/kg daily for 4 months [29] or at 10 or 15 mg/kg body weight for seven successive days [30]. Unfortunately, pharmacokinetic and pharmacodynamic studies of TPEN in humans are still lacking. Therefore, further studies are needed to establish if TPEN can be therapeutically applied to patients with leukemia.

How does TPEN provoke apoptosis in B-ALL? Mounting evidence suggests that TPEN induces cell death by generation of H2O2 by acting as class 5 mitocan [31] or by forming a TPEN-copper complex [19]. In both incidences, TPEN chelates active metals such as iron and copper from mitochondrial complex I-III to convert oxygen (O2) into anion superoxide radicals (O2.-), which in turn dismutate into H2O2. Whatever the mechanism, we found a significantly increased in the oxidized OS-sensor protein DJ-1 as a probe of the specific and selective oxidation residue Cys106-SH (thiol) of DJ-1 into Cys106-SO3 (sulfonate) by H2O2 [32]. In addition, H2O2 can also indirectly trigger the activation of the transcription factor TP53 through MAPK kinases [33]. Consequently, TP53 activates PUMA [34,35]. Effectively, we observed a significantly increased in both proteins TP53 and PUMA according to flow cytometry. Taken together these observations suggest that TPEN/H2O2 induce apoptosis via activation of TP53 and PUMA. Outstandingly, PUMA represents one of the most potent initiator pro-apoptotic BH3-only proteins [36]. Indeed, PUMA cooperates with direct activator proteins to promote mitochondrial outer

membrane permeabilization [37] and activation of caspases, e.g., CASPASE-3, which play a central role in the execution-phase of cell apoptosis [38]. In this regard, we have consistently detected an association of increased positive cells CD34/CD19/CASP3 with positive cells CD34/CD19/DJ-1Cys106-SO3. These data suggest that stressed CD34+/CD19+ cells by TPEN/H2O2 ended up dismantled by CASP3. Interestingly, CASP3+ and PUMA+ showed similar percentage of activation in CD34+/CD19+ cells. Taken together, these observations imply that TPEN induces apoptosis in a cascade-like mechanism: TPEN/H2O2 >>> TP53> PUMA>> CASP3 >cell death.

4. Materials and Methods

4.1.   Experiments with whole bone marrow samples from chemoresistant leukemia pediatric patients

Whole bone marrow (WBM) samples were collected from de novo B-ALL (n=5) and refractory B-ALL (n=6) pediatric patients, and diagnosed according to conventional karyotyping and classical immunophenotypical criteria. Written informed consent of the admitted patients at the Pablo Tobon Uribe Hospital (HPTU), Medellin-Colombia was obtained in accordance with the Ethics Committee for Research Act # 17-10-697 (May 17th, 2017) and HPTU 08/2018, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Total BM samples (100 µL) were plated in 96-well plaques and incubated without or with TPEN (50–500 µM or 50 µM) for 24 h at 37 °C in a humidified atmosphere of 5% CO2.

4.2.   The simultaneous analysis of CD34, CD19, and CASPASE-3 or PUMA by flow cytometry in bone marrow (BM) cells from chemoresistant leukemia pediatric patients

Flow cytometry acquisition was used to determine the percentage of CD34/CD19/CASPASE−3 triple-positive cells. After each treatment with or without TPEN, erythrocytes were lysed and BM cells were washed with PBS and permeabilized with 0.2% triton X-100 plus 1.5% bovine serum albumin (BSA) for 30 min and simultaneously incubated for 20 min at 37 °C in the dark with mouse anti-human CD34-PE and CD19-PEcy7 antibodies (1:200, BD Biosciences) and caspase-3 (Rabbit, Millipore, cat # AB3623) or PUMA (Rabbit, Abcam, cat ab-9643). Subsequently, the cells were washed and incubated with (1:500) Dylight donkey anti-rabbit (488 nm, cat # DI-XXX). Cells were then analyzed using a BD LSRFortessa II flow cytometer (BD Biosciences), and 20,000 events were acquired for analysis. Quantitative data and figures were obtained using FlowJo 7.6.2 Data Analysis Software.

4.3. Detection of oxidized-(Cys106) DJ-1/ CASPASE-3 and PUMA/TP53 by flow cytometry in bone marrow (BM) cells from chemoresistant leukemia pediatric patients

After each treatment with or without TPEN, RBC were lysed and WBM cells were washed with PBS and permeabilized with 0.2% triton X-100 plus 1.5% bovine serum albumin (BSA) for 30 min, cells were washed and incubated with anti-p53 (Millipore, catMA5-12453), PUMA (Abcam, cat # ab-9643), caspase-3 (Rabbit, Millipore, cat # AB3623) and oxidized DJ-1 (spanning residue C106 of Human PARK7/DJ1; oxidized to produce cysteine sulfonic (SO3) acid). Mouse, Millipore, cat # Millipore, cat # MABN1773) primary antibodies (1:500, diluted in PBS containing 0.1% BSA). Subsequently, the cells were washed and incubated with (1:500) Dylight donkey anti-rabbit (594 nm, cat # DI-1094) or -mouse (488 nm, cat # DI-2488) secondary antibodies for 30 min. at RT in the dark. After washing with PBS, the cells were suspended in 500 µL of PBS. Analysis was performed on a BD LSRFortessa II flow cytometer (BD Biosciences).

Cells without primary antibodies served as negative control. For assessment, it was acquired 20,000 events and quantitative data and figures were obtained using FlowJo 7.6.2 Data Analysis Software.

4.4 Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6 scientific software (GraphPad, Software, Inc. La Jolla, CA, USA). One-way ANOVA with a Tukey post hoc and Student t tests was used to compare the differences between the experimental groups. A P-value <0.05 (*), <0.01 (**) and <0.001 (***) was considered statistically significant.

5. Conclusions

We have demonstrated that TPEN as pro-oxidant agent is capable of triggering apoptosis in ex vivo whole bone marrow cells from B-ALL pediatric patients independent of diagnosis (e.g., de novo and refractory B-ALL) and sex through a similar OS mechanism previously shown in Jurkat cell model of ALL [25] as well as ex vivo peripheral blood-isolated ALL cells [27]. Our present data support the view that TPEN can be safely used in the clinic as salvage therapy for acute lymphocytic leukemia.

Author Contributions:

Conceptualization, C.V.-P. and M.J.-del-R.; **methodology**, M.M.-P.; **validation**, C.V.-P. and M.M.-P.; **formal analysis**, C.V.-P., M.M.-P. and M.J.-del-R.; **investigation**, C.V.-P., M.M.-P., L.M.Q.-D, A.R.-R., N.A.V.-Z. and M.J.-del-R.; **data curation**, M.M.-P. and M.J.-del-R.; **writing—original draft preparation,** C.V.-P. and M.J.-del-R.; **writing—review and editing**, S.K., C.S.V.; **supervision,** C.V.-P., M.M.-P., L.M.Q.-D, A.R.-R., N.A.V.-Z. and M.J.-del-R.; **project administration**, M.J.-del-R.; **funding acquisition**, C.V.-P., **whole bone marrow samples and clinical data acquisition from** *de novo* **and refractory B-ALL patients,** L.M.Q.-D, A.R.-R., and N.A.V.-Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by "Fundación Alfonso Moreno Jaramillo" grant #2017–16748 and grant #2018–20454.

Institutional Review Board Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee for Research Act # # 17-10-697 and HPTU 08/2018 from HPTU, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Statement: Informed consent was obtained from pediatric ex vivo B-ALL collected in the study.

Data Availability Statement: All data used during the study appear in the submitted article.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role

Acknowledgments: The authors thank "Fundación Alfonso Moreno Jaramillo" for financial support.

Conflicts of Interest: The authors declare no conflict of interests.

Sample Availability: Not available.

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