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# Piperlongumine inhibits the proliferation and survival of B-cell acute lymphoblastic leukemia cell lines irrespective of glucocorticoid resistance

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ABSTRACT

Piperlongumine (PL), a pepper plant alkaloid from *Piper longum*, has anti-inflammatory and anti-cancer properties. PL selectively kills both solid and hematologic cancer cells, but not normal counterparts. Here we evaluated the effect of PL on the proliferation and survival of B-cell acute lymphoblastic leukemia (B-ALL), including glucocorticoid (GC)-resistant B-ALL. Regardless of GC-resistance, PL inhibited the proliferation of all B-ALL cell lines, but not normal B cells, in a dose- and time-dependent manner and induced apoptosis via elevation of ROS. Interestingly, PL did not sensitize most of B-ALL cell lines to dexamethasone (DEX). Only UoC-B1 exhibited a weak synergistic effect between PL and DEX. All B-ALL cell lines tested exhibited constitutive activation of multiple transcription factors (TFs), including AP-1, MYC, NF-κB, SP1, STAT1, STAT3, STAT6 and YY1. Treatment of the B-ALL cells with PL significantly downregulated these TFs and modulated their target genes. While activation of AURKB, BIRC5, E2F1, and MYB mRNA levels were significantly downregulated by PL, but SOX4 and XBP levels were increased by PL. Intriguingly, PL also increased the expression of p21 in B-ALL cells through a p53-independent mechanism. Given that these TFs and their target genes play critical roles in a variety of hematological malignancies, our findings provide a strong preclinical rationale for considering PL as a new therapeutic agent for the treatment of B-cell malignancies, including B-ALL and GC-resistant B-ALL.



## 1. Introduction

ALL is the most common childhood cancer, accounting for approximately 30% of all childhood malignancies in North America [1]. Although the majority of children with ALL are cured, relapsed ALL remains a leading cause of cancer-related deaths in children [2]. Approximately 20% of patients become resistant to GC during the treatment of B-ALL and up to 80% of relapsed patients are classified as being GC-resistant [3]. Despite the critical importance of developing new treatments for childhood ALL, especially GC-resistant ALL, many factors impede drug discovery and development.

Herbal remedies based on medicinal or dietary plants are becoming increasingly popular in the world [4]. Various phytochemicals, which are reported to regulate molecular signal transduction pathways capable of inducing cell death, are now a major focus of research. Importantly, many FDA-approved anticancer therapeutic agents are derived from phytochemicals. Paclitaxel, one of the newest FDA-approved anticancer therapeutic agents, was isolated from the bark of Pacific Yew trees [5].

Piperlongumine (PL), one of the primary constituents isolated from the fruit of the long pepper, *Piper longum* Linn [6], has antitumor, antioxidant, antibacterial, antifungal and antiinflammatory properties [7]. Interest in PL as a potential cancer drug has drawn attention since Raj et al. [8] reported that PL selectively kills human solid tumor cells, but not their normal non-transformed counterparts. We previously reported that PL selectively kills Burkitt lymphoma (BL) cells by inhibiting the LMP1-NF-κB-MYC axis and their downstream target genes [9,10]. To extend our

*Abbreviations:* PL, piperlongumine; ALL, acute lymphoblastic leukemia; GC, glucocorticoids; DEX, dexamethasone; TF, transcription factor; TBE, trypan blue exclusion; BSO, buthionine sulfoximine; DTT, dithiothreitol; PBMC, peripheral blood mononuclear cell; NE, nuclear protein extract; EMSA, electrophoretic mobility shift assay; EBV, Epstein Barr virus; GSH, glutathione; ROS, reactive oxygen species.

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research on hematological malignancies, we investigated the effect of PL and its underlying molecular mechanisms, focusing on TFs and their downstream target genes, on the growth and survival of B-ALL and GC-resistant B-ALL.

## 2. Materials and methods

### 2.1. Chemicals and B-ALL cell lines

PL was purchased from INDOFINE (Hillsborough, NJ), dissolved in dimethyl sulfoxide (DMSO). Dexamethasone (DEX), Buthionine sulfoximine (BSO), dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO) and dissolved in Ethanol or distilled water. Five GC-resistant B-ALL cell lines, HAL-01, RCH-ACV, Reh, SEM and UoC-B1 and four GC-sensitive B-ALL cell lines, 697, NALM6, RS;11 and SUP-B15 were obtained from Dr. Pufall (Univ. of Iowa) and maintained, at 37 °C and 5% $CO_2$, in RPMI 1640 medium with 10% fetal bovine serum. Human peripheral blood mononuclear cell (PBMC) B cells were isolated from leukoreduction system chambers generated by the University of Iowa Blood Center using centrifugation through a Ficoll-Hypaque cushion and CD45R (B220) microbeads and MACS® separation columns (Miltenyi, Auburn, CA).

### 2.2. Cell growth and proliferation

Growth and proliferation of B-ALL cells were determined with the Cell Titer 96 MTS/PMS assay (Promega, Madison, WI). Briefly, $1 \times 10^5$ cells were re-suspended in 100 μl growth medium and plated into 96-well plates (Costar, Cambridge, MA). 20 μl MTS/PMS solution was added to each well 4 h before measurement at the absorbance at 490 nm using a Multiskan Spectrum 96-well plate spectrophotometer (Thermo Scientific, Hudson, NH).

### 2.3. Apoptosis

Programmed cell death was evaluated with the help of the trypan blue exclusion (TBE) and DNA fragmentation assays. For both assays, $10 \times 10^6$ cells were treated with PL(at $IC_{50}$) or left untreated for 24 h at 37 °C and 5% $CO_2$. Cells were harvested and resuspended in PBS. For TBE assay, 4 parts of 0.4% trypan blue solution were mixed to 1 part of cell suspension, and the proportion of blue-staining (dead) cells was determined using a hemocytometer. Results are expressed as a percentage of the number of live cells divided by the number of total cells. For DNA fragmentation assay, DNA was extracted using the Puregene Cell kit (Gentra Systems, Minneapolis, MN) followed by electrophoretic fractionation on 1.0% agarose gels containing ethidium bromide.

### 2.4. Preparation of nuclear extract

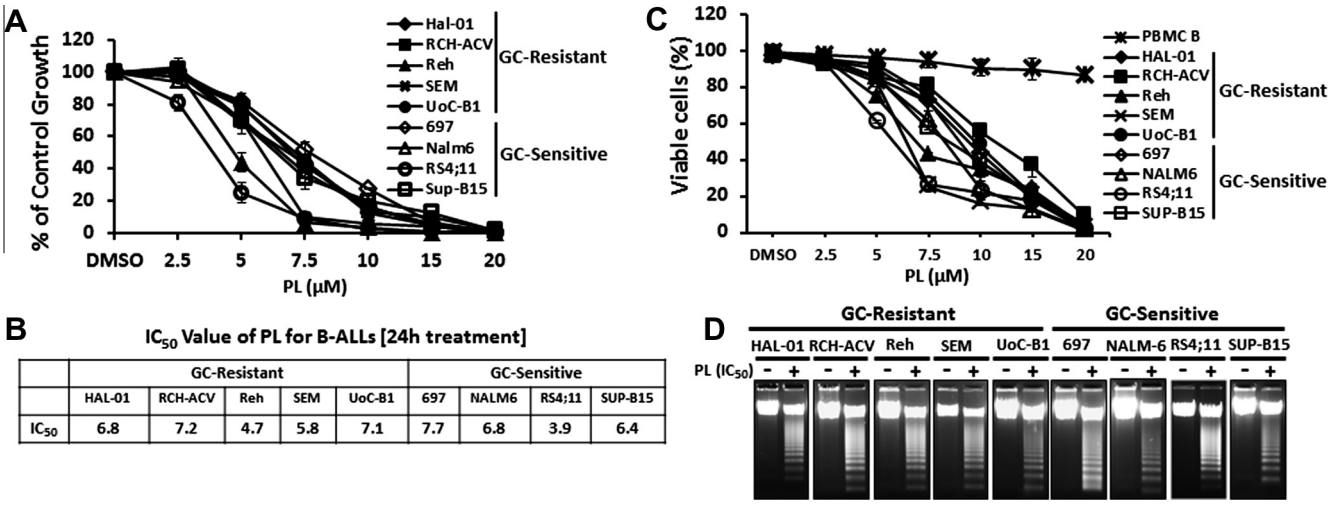
Pellets of $10 \times 10^6$ cells were lysed in 400 μl buffer A (10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT and 0.2 mM PMSF) at 4 °C for 10 min. Lysate was centrifuged for 5 min at 14,000×*g* and supernatant was stored at −70 °C as cytosolic extract. The residual pellet was resuspended in 100 μl of ice-cold buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 20% [v/v] glycerol, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF). After incubation at 4 °C for 20 min, the lysate was centrifuged for 6 min at 14,000×*g* and the supernatant was stored at −70 °C as nuclear extract (NE). Protein concentrations of NE were determined with the help of the BCA kit (Bio-Rad, Richmond, CA).

### 2.5. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out in a final volume of 25 μl binding buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% [w/v] glycerol, 0.1 mg/ml sonicated salmon sperm DNA), 10 μg nuclear extract, and radiolabeled oligonucleotide that contained consensus AP-1, MYC, NF-κB, p53, SP1, STAT1, STAT3, STAT4, STAT6 or YY1 binding site (Santa Cruz Biotechnology, Santa Cruz, CA). Oligonucleotide was end-labeled to a specific activity of $10^5$ CPM using γ-[$^{32}$P]-ATP and T4-polynucleotide kinase and purified on a Nick column (GE Healthcare, Piscataway, NJ). Reaction mixtures were incubated at room temperature for 20 min and then loaded on 6% non-denaturing polyacrylamide gels after 2 μl of 0.1% bromophenol blue was added. Gels were dried and subjected to autoradiography.

### 2.6. Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) assays

For RT, total RNA was extracted using the TRIzol (Sigma–Aldrich, St. Louis, MO) and cDNA synthesis with 1 μg RNA and




| | GC-Resistant | | | | | GC-Sensitive | | | |
|---|---|---|---|---|---|---|---|---|---|
| | HAL-01 | RCH-ACV | Reh | SEM | UoC-B1 | 697 | NALM6 | RS4;11 | SUP-B15 |
| $IC_{50}$ | 6.8 | 7.2 | 4.7 | 5.8 | 7.1 | 7.7 | 6.8 | 3.9 | 6.4 |

**Fig. 1.** PL induced growth inhibition and apoptosis of all B-ALL cell lines regardless of GC-resistance. (A) Cells ($1 \times 10^6$/ml) were treated with PL as indicated for 24 h. Proliferation was measured by MTS assay as described at Section 2. (B) The $IC_{50}$ concentration of PL for each B-ALL cell lines. Apoptosis was measured by TBE assay (C) and DNA fragmentation assay (D). Cells ($5 \times 10^5$/ml) were treated with PL as indicated for 24 h and then subjected to TBE or DNA fragmentation assay as described at Section 2. Error bars shows the standard deviation from data represent the mean of triplicate determinations.

the AMV reverse transcriptase kit from Roche (Indianapolis, IN). qPCR relied on the TaqMan universal PCR master mix from Applied Biosystems (Carlsbad, CA) and – after 1 cycle of 50 °C for 2 min and 1 cycle of 95 °C for 10 min – 40 cycles of DNA amplification at 66 °C (1 min) and 95 °C (15 s). Internal probes were labeled with the fluorescent reporter dye, 6-carboxyfluorescein (6-FAM), on the 5′ end and the quencher dye, Black Hole (BHQ), on the 3′ end. The sequence of gene-specific PCR primers listed in Supplementary Table 1. Amplicons were generated and analyzed using the Applied Biosystems 7900 HT device and ABI SDS v 2.3 software (Applied Biosystems, Carlsbad, CA), respectively. Gene expression was compared to Hprt message, which was used as control. Gene expression levels in BL cells were compared to expression levels in human PBMC B cells and presented as fold gene expression change.

## 3. Results

### 3.1. PL inhibits proliferation of B-ALL cell lines irrespective of GC-resistance

To evaluate whether PL inhibits the proliferation of B-ALL cells, five GC-resistant B-ALL cell lines (HAL-01, RCH-ACV, Reh, SEM and UoC-B1) and four GC-sensitive B-ALL cell lines (697, NALM6, RS;11 and SUP-B15) were treated with various concentrations of PL (2.5–20 μM) for 24 h or 48 h and then cell proliferation was quantified using the MTS assay. As shown in Fig. 1A, PL inhibited the growth of all cell lines in a dose- and time-dependent (Supplementary Fig. 1) manner, irrespective of GC-resistance. The $IC_{50}$ values for PL ranged between 3.9 μM and 7.7 μM (Fig. 1B) and, in subsequent experiments, PL was used at the $IC_{50}$ specific for each cell line.

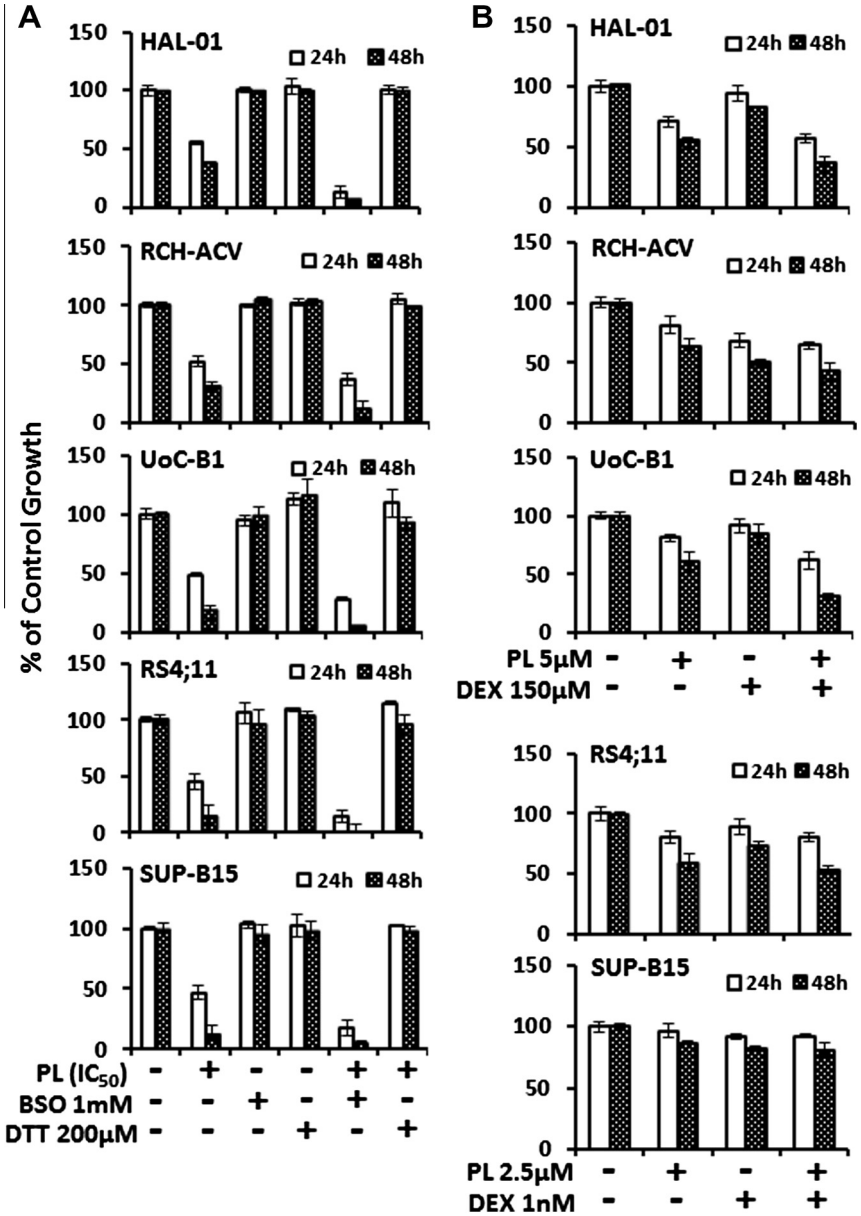



**Fig. 2.** (A) PL caused apoptosis by elevation of ROS. Cells ($1 \times 10^6$/ml) were treated with PL($IC_{50}$), 1 mM BSO or 200 μM DTT individually or combination of them as indicated for 24 h or 48 h. (B) PL sensitizes only UoC-B1 cell line to DEX but not in others. Cells ($1 \times 10^6$/ml) were treated with low dose of PL or DEX depending on each cell line's susceptibility individually or combination of them as indicated for 24 h or 48 h. Proliferation was measured by MTS assay as described at Section 2. Error bars shows the standard deviation from data represent the mean of triplicate determinations.

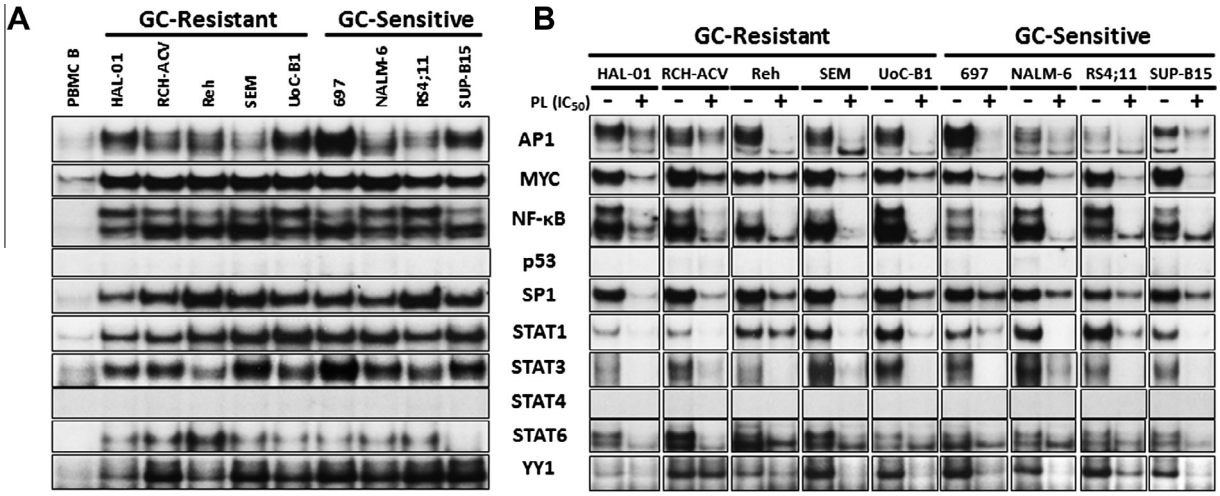



**Fig. 3.** (A) Status of DNA-binding activity of TFs and (B) PL-induced repression of constitutively activated TFs in B-ALL cells. EMSA was performed with NE from each B-ALL cell line ($1 \times 10^6$/ml) in the absence or presence of PL($IC_{50}$) for 24 h as described at Section 2.

### *3.2. PL selectively induced apoptosis of human B-ALL cell lines, not normal B cells*

We then examined whether PL selectively kills B-ALL cells relative to normal PBMC B cells. B-ALL cells and normal PBMC B cells were treated for 24 h with PL (2.5–20 μM) and the proportion of viable cells was determined using the TBE assay. While PL effectively killed B-ALL cells in a dose-dependent manner (Fig. 1C), normal PBMC B cells were largely unaffected by the drug. Apoptotic cell death of the PL-treated B-ALL cells was confirmed by DNA fragmentation analysis (Fig. 1D). These results demonstrated that PL selectively killed B-ALL cells, but not normal PBMC B cells through apoptosis.

### *3.3. PL causes apoptosis by elevating ROS via modulating GSH level*

PL can induce apoptosis in cancer cells by reducing glutathione (GSH) levels, which results in elevated levels of reactive oxygen species (ROS) [8]. Consequently, we next investigated the effect of PL on GSH and ROS in B-ALL cells. Inhibition of GSH by BSO, which blocks GSH synthesis, synergistically increased PL-mediated growth inhibition in B-ALL cells. However, treatment with DTT, which maintains reduced GSH levels, completely abolished the inhibitory effect of PL (Fig. 2A and Supplementary Fig. 2A). Thus, we confirmed that PL induces apoptosis of B-ALL cells by increasing ROS through regulating GSH level.

### *3.4. PL does not sensitize majority of B-ALL cells to DEX*

Next, we examined whether PL can sensitize B-ALL cells, particularly GC-resistant cells, to DEX. B-ALL cells were treated with PL and DEX, alone or in combination, at low doses that cause only a modest growth inhibition when used as single agents. As shown in Fig. 2B and Supplementary Fig. 2B, a weak synergistic effect between PL and DEX was only observed in UoC-B1 cells, but not in other B-ALL cell lines. This result demonstrates that PL does not sensitize B-ALL cells to DEX. The effect of DEX on the proliferation of B-ALL cells is presented in Supplementary Fig. 3.

### *3.5. PL represses constitutive activation of various TFs that play critical role in the proliferation of normal and neoplastic B-lymphocytes*

The aberrant deregulation of TF(s) is associated with various types of cancer [11]. To test whether the proliferation and survival of B-ALL is a result of constitutive activation of TF(s), we investigated the DNA-binding activity of the following TFs in B-ALL cells using EMSA: AP-1, MYC, NF-κB, p53, SP1, STAT1, STAT3, STAT4, STAT6 and YY1. All B-ALL cell lines exhibited constitutive activation of AP-1, MYC, NF-κB, SP1, STAT1, STAT3, STAT6 and YY1, whereas p53 and STAT4 showed basal levels or no binding activity compared to the normal, control PBMC B cells (Fig. 3A). This result led us to investigate whether PL inhibits the constitutive activation of these TFs. As shown in Fig. 3B, PL dramatically inhibited the constitutive activation of TFs in all B-ALL cell lines. Interestingly, the basal level of DNA-binding activity of p53 and STAT4 did not change with PL treatment. These results suggested that PL inhibits the constitutive activation of multiple TFs critical for the proliferation and survival of B-ALLs.

### *3.6. PL changes expression of target genes of these TFs*

Next, we evaluated the expression of fifty genes previously implicated as targets of these TFs to identify which genes were involved in PL-induced apoptosis. AURKB/Aurora kinase B, BIRC5/Survivin, E2F1 and MYB were significantly downregulated in B-ALL cells (Fig. 4A) after PL($IC_{50}$) treatment for 24 h. In contrast, expression of SOX4, XBP1 and CDKN1A/p21 were upregulated (Fig. 4B) with PL($IC_{50}$) treatment. Next, we used qPCR and EMSA to determine whether the elevated expression of p21 in the presence of PL was dependent on p53. Interestingly, we did not find any change in the level of p53 mRNA (Fig. 4B) or DNA-binding activity of the protein (Fig. 4C) by PL treatment. These results indicate that the elevation of p21 is independent of p53. Somewhat unexpectedly, BCL family genes, including BAX, BCL2, and BCL2A1/BFL1, were not affected by PL though BCLxL and BCL2A11/BIM exhibited insignificant elevation compared to normal PBMC B cells (Supplementary Fig. 4). These results indicate that PL-mediated suppression of the TFs results in the modulation of target genes known to be important for the proliferation and survival of B-ALL cells.

## 4. Discussion

Although more than 85% of children with ALL are cured, relapsed and/or nonresponsive ALL remains a leading cause of cancer-related death in children and young adults [2]. Despite the promising potential of PL in cancer therapy, its effect on the growth

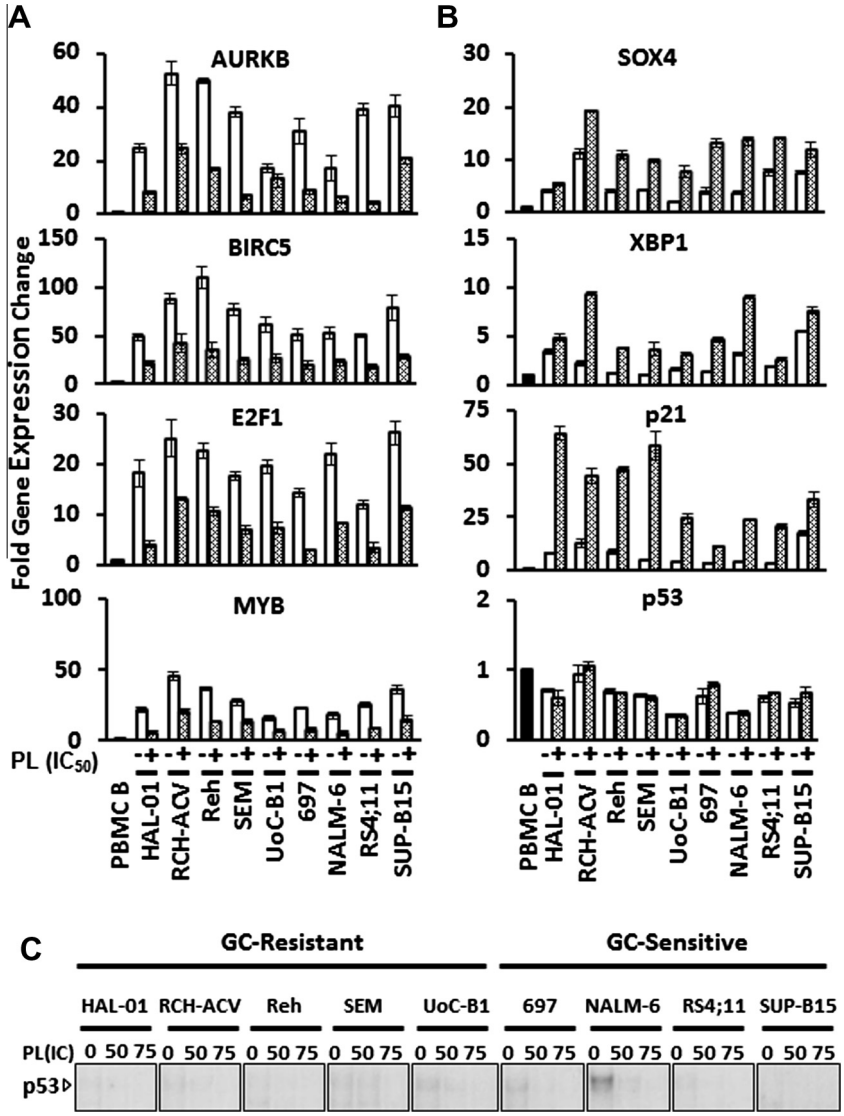



**Fig. 4.** PL disrupts the expression of target genes of these TFs. (A) Upregulated genes by PL. (B) Downregulated genes by PL. mRNA level of each gene in the absence or presence of PL twere analyzed using qPCR as described at Section 2. Data were normalized to Hprt and converted to fold gene expression change by dividing the normalized value from each cell line or by that of normal PBMC B cells. Error bars represent the standard deviation from a representative experiment performed in triplicate. (C) Basal level of p53 DNA-binding activity was not affected by PL treatment. EMSA was performed with NE from each B-ALL cell line ($1 \times 10^6$/ml) in the absence or presence of PL($IC_{50}$) for 24 h as described at Section 2.

and survival of B-ALL, especially GC-resistant B-ALL, were unknown and the focus of this work. We found that PL inhibited growth of B-ALL cell lines in a dose- and time-dependent manner regardless of GC-resistance ($IC_{50}$ of 3.9–7.7 μM). This result is consistent with our recent report that the $IC_{50}$ range for PL in human BL cell lines is 2.8–8.5 μM [9] and is 5.1–9.0 μM for mouse BL cell lines [10]. Interestingly, no difference in susceptibility to PL between GC-resistant and -sensitive cell lines was observed. In line with our recent findings for BL, PL selectively killed B-ALL cells, but not normal PBMC B cells [9,10]. Similarly, in accordance with previous reports [8,9], we also confirmed in B-ALL cells that PL causes growth inhibition and apoptosis through elevation of ROS. Since the biggest hurdle to treat B-ALL patients is the increase of GC-resistance and relapse of B-ALL cells [2,3], we evaluated whether PL can sensitize B-ALL cells, especially GC-resistant cells, to DEX. Although GC-resistant cell lines, HAL-01 or UoC-B1 exhibited additive or weak synergistic growth inhibition, the other cell lines did not. These results demonstrate that PL does not sensitize most B-ALL cell lines to DEX.

Deregulation of signal transduction pathways are implicated in the development of cancer [5]. We determined that AP1, MYC, NF-κB, SP1, STAT1, STAT3, STAT6 and YY1 are activated in B-ALL compared to normal B-cells. Remarkably, PL repressed the constitutive activation of these TFs. Activation of these TFs in a variety of tumors including leukemia is consistent with reports from the literature [12–15] and inhibition of these constitutively activated these TFs can lead to apoptosis [12,13,15]. Moreover, numerous reports demonstrate that TFs act coordinately in the development of cancer [9,10,15,16], which supports our finding that PL causes apoptosis in B-ALL cells by inhibiting a network of TFs, rather than the suppression of one or two TF(s).

Since TFs have the ability to alter global transcriptional profiles [17], we validated a set of 50 target genes by qPCR. AURKB/Aurora kinase B, BIRC5/Survivin, E2F1, and MYB exhibited elevated levels in B-ALL cells relative to normal B-cells and were significantly downregulated by PL. AURKB [18] and Survivin [19] are overexpressed in many cancers and inhibition of these proteins results in apoptosis [20]. E2F1 is a direct target gene of MYC and important

for NF-κB-dependent survival of tumor cells [21]. MYB, a direct target of NF-kB [22], is a transcriptional activator whose abrogation in cancer cells causes apoptosis [23]. In contrast, PL significantly upregulated SOX4, XBP1 and p21. Several reports support the potential role of these genes in PL-mediated apoptosis. For example, SOX4 is overexpressed in an ATM/ATR-dependent but p53-independent manner [24] and activates apoptosis [17]. Interestingly, SOX4 can directly repress NF-κB p50 at transcriptional level [25] and also interacts with a number of TFs to synergistically activate proapoptotic and antiproliferative SOX4 target genes [26]. NF-κB can regulate XBP-1 by inhibition of miR-214, which is a negative regulator of XBP-1 [27]. Conversely, Xbp1 suppresses intestinal tumorigenesis by inhibition of NF-κB and STAT3 [28]. Capsaicin [29] killed cancer cells by increase of XBP1 at the transcriptional or protein level. Consequently, in accordance with these reports, the genes identified in this work are likely to be critical downstream targets of the altered TF profile induced by PL.

Interestingly, p21 was elevated in B-ALL cell lines compared to normal B-cells and its expression was further augmented by PL in a p53-independent mechanism. Considering the accumulation of evidence for a p53-independent role of p21 in cancer [30], activation of p21 is not unexpected in B-ALL. NF-κB-mediated activation of p21 is critical to avoid p53-induced apoptosis and the initiation and maintenance of leukemogenesis [31]. RelB [32] and STATs, including STAT3 [33], regulate the oncogenic function of p21 at the transcriptional level [32]. In accordance with these reports, constitutive activation of NF-κB and STAT3 in B-ALL cells is likely driving this anti-apoptotic program. However, PL increased p21 expression without a change in p53 status. In fact, the level of p53 DNA-binding activity was unchanged even at a higher dose of PL($IC_{75}$). Although activation of p21 is often regulated by p53, p21 can act as a master effector of several tumor suppressor pathways in a p53-independent manner. Depending on the cellular context and circumstances, p53-independent activation of p21 as a tumor repressor is controlled by several TFs, including SP1 and STATs [33]. Therefore, our finding that PL induces a p53-independent activation of p21 suggests that the function of p21 may be switched from antiapoptotic to proapoptotic by treatment with PL. However, the precise mechanism of how this switch in p21 function occurs needs to be investigated in further detail.

In summary, PL causes growth inhibition and apoptosis in B-ALL cells, irrespective of GC-resistance, by downregulation of multiple, constitutively activated TFs, including AP1, MYC, NF-kB, SP1, STAT1, STAT3 STAT6 and YY1. Although further studies are required to fully understand the mechanism of PL-induced B-ALL apoptosis, the results here demonstrate that PL could be a new therapeutic agent for the treatment of malignant B-cell tumors including B-ALL and GC-resistant B-ALL.

## Competing interests

Authors declare no competing interests.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.131.

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