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Piperlongumine inhibits proliferation and survival of Burkitt lymphoma *in vitro*

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Abstract

Piperlongumine (PL), a pepper plant alkaloid from *Piper longum*, kills solid tumor cells in a highly selective, potent fashion. To evaluate whether PL may have similar effects on malignant blood cells, we determined the efficacy with which PL inhibits the B-lymphocyte derived neoplasm, Burkitt lymphoma (BL). Low micromolar concentrations of PL ($IC_{50} = 2.8 \times 8.5 \mu M$) curbed growth and survival of two EBV⁺ BL cell lines (Daudi, Raji) and two EBV⁻ BL cell lines (Ramos, DG-75), but left normal peripheral blood B-lymphocytes unharmed. PL-dependent cytotoxicity was effected in part by reduced NF- κ B and MYC activity, with the former being caused by inhibition of I κ B α degradation, nuclear translocation of p65, and binding of NF- κ B dimers to cognate DNA sequences in gene promoters. In 4 of 4 BL cell lines, the NF- κ B/MYC-regulated cellular target genes, *E2F1* and *MYB*, were down regulated, while the stress sensor gene, *GADD45B*, was up regulated. The EBV-encoded oncogene, *LMP-1*, was suppressed in Daudi and Raji cells. Considering that NF- κ B, MYC and LMP-1 play a crucial role in the biology of many blood cancers including BL, our results provide a strong preclinical rationale for considering PL in new intervention approaches for patients with hematologic malignancies.

Keywords

Long pepper; pyridone alkaloid; B-cell malignancy; non-Hodgkin lymphoma; cancer therapy and prevention

1. Introduction

Herbal remedies based on medicinal or dietary plants and fruits from India, China and other Asian countries are becoming increasingly popular in the Western world including the

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Conflict of Interest

The authors declare that they have no competing interests to report.

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United States [1]. An important underlying reason for this development is evidence that phytochemicals isolated from these plants and/or their fruits, notably including peppers, exhibit significant health-promoting activity [2]. Among the most potent phytochemicals of this sort is piperlongumine (PL, Fig. 1A), a pyridone alkaloid found in the fruit of the long pepper, *Piper longum* Linn [3]. PL has recently been demonstrated to have beneficial effects on a plethora of pathophysiologic pathways involved in the natural history of major human diseases; e.g., oxidative stress [4], inflammation [5], hemostasis [6], hyperlipidemia [7], dementia [8] and pain [9]. Furthermore, PL inhibits infectious pathogens (e.g., *amoebae* [10], schistosoma [11], fungi [12]) or their insect vectors (e.g., mosquitoes at larval [13] or adult [14] stages of development); protects tissues, such as liver, from damage induced by toxic chemicals [15] or ionizing radiation [16]; and, of importance for cancer research, hampers the growth and dissemination of neoplastic cells *in vitro* [17] and *in vivo* [18].

The interest in PL as a potential cancer drug experienced a quantum leap very recently when a high-profile study demonstrated that the compound selectively killed 13 different human solid tumor cells (including melanoma and bladder, breast and lung cancer) but left their normal counterparts essentially unscathed [19]. Using high-throughput screenings of several thousand biologically active compounds in a human osteosarcoma cell line that harbored a p53-responsive luciferase reporter as indicator of programmed cell death, the investigators found that PL was the strongest and most consistent inducer of the pro-apoptotic transcription program among all compounds tested. Subsequent proteomic analysis of putative PL-binding proteins in tumor cells led to the identification of glutathione-S-transferase-P1 (GSTP1) and carbonyl reductase 1 (CBR1) – two enzymes involved in cellular responses to oxidative stress. This suggested that PL-mediated killing of cancer cells relies in large measure on a compromised defense against reactive oxygen species (ROS). Consistent with that theory, treatment with PL resulted in increased ROS levels in cancer cell lines but not in immortalized cell lines that had not yet completed malignant transformation [19].

To extend the above-described studies from solid to blood cancers and begin with the evaluation of the nuclear factor kappa-B (NF- κ B)/myelocytomatosis oncogene (MYC) pathway in PL-dependent cell killing, we here decided to determine the effects of PL on the B cell-derived neoplasm, Burkitt lymphoma (BL). Employing two EBV⁺ cell lines, Daudi and Raji, and two EBV⁻ cell lines, Ramos and DG-75, as principal experimental model system, we found that PL curbed the proliferation and survival of BL cells in the same low micromolar concentration range shown to be effective in carcinoma cells [19]. EMSA studies demonstrated that treatment of BL with PL results in a significant drop in NF- κ B/MYC activity, which leads, in turn, to changed expression levels of the cellular target genes, *E2F1*, *MYB* and *GADD45B*, and, in EBV⁺ cells, down regulation of the viral oncogene, *LMP-1*. Our results provide encouragement for including PL in new treatment and prevention strategies for BL and related hematologic malignancies of the B-cell lineage.

2. Materials and methods

2.1. Cells and pharmacological inhibitors

BL cell lines, DG-75, Daudi, Raji and Ramos were purchased from ATCC (Manassas, VA) and maintained at 37 °C and 5% CO₂ in ATCC-formulated RPMI 1640 basic cell culture medium supplemented with 10% heat-inactivated fetal bovine serum. Normal B-lymphocytes were isolated from human peripheral blood mononuclear cells (PBMCs) using centrifugation through a Ficoll-Paque density gradient medium (30 min, 400 g) followed by positive, magnetic microbead-assisted fractionation on CD45R (B220) MACS® cell separation columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. PBMCs were obtained from discarded leukoreduction system chambers kindly

provided by the University of Iowa DeGowin Blood Center. PL was purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ), dissolved in dimethyl sulfoxide (DMSO), and added to BL cells such that the final concentration of DMSO did not exceed 0.1% v/v. Buthionine sulfoximine (BSO) and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in sterile, distilled water.

2.2. Cell growth and proliferation

Growth and proliferation of BL cells were determined with the assistance of the Cell Titer 96 MTS/PMS assay (Promega, Madison, WI). Briefly, 3×10^4 cells were re-suspended in 100 μ l growth medium and plated into 96-well plates (Costar, Cambridge, MA). Twenty hours later, 20 μ l MTS/PMS solution was added to each well. Cells were incubated for another 4 hours before the absorbance at 490 nm was measured 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, 10^7 cells were treated with PL (at IC_{50}) or left untreated for 24 hours 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. 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 whole cell extracts and Western blot analysis

Pellets of 10^7 cells were resuspended and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, 1 μ M sodium orthovanadate) at 4 °C for 30 minutes. Lysates were centrifuged for 6 minutes at $14,000 \times g$ and supernatants were stored at -70 °C as whole cell extracts. Total protein concentrations were determined using a BCA kit (Bio-Rad, Richmond, CA). Western blots were performed with 40 μ g total protein resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with antibodies (Abs) to: Caspase-3 (9662; Cell Signaling, Danvers, MA); p65 (sc-109) and I κ B α (sc-847; both from Santa Cruz Biotechnology, Santa Cruz, CA); or β -actin (A5316; Sigma-Aldrich, St. Louis, MO). Proteins were visualized using horseradish peroxidase-conjugated secondary Ab (1:5000) and the ECL detection kit from Amersham (GE Healthcare, Piscataway, NJ).

2.5. Preparation of nuclear and cytosolic extracts

Pellets of 10^7 cells were lysed in 400 μ l 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 minutes. Lysate was centrifuged for 5 minutes at $14,000 \times g$ and supernatant was stored at -70 °C as cytosolic extract (CE). The residual pellet was resuspended in 100 μ l 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 minutes, the lysate was centrifuged for 6 minutes at $14,000 \times g$ and the supernatant was stored at -70 °C as nuclear extract (NE). Protein concentrations of CE and NE were determined with the help of the BCA kit (Bio-Rad, Richmond, CA).

2.6. Electrophoretic mobility shift assays (EMSAs) and super-shift assays

EMSA was carried out in a final volume of 25 ml 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 μ g nuclear extract, and oligonucleotide that contained either consensus NF- κ B (Promega, Madison, WI) or Myc-Max binding sites (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 with radiolabeled oligonucleotides were incubated at room temperature for 20 minutes and then fractionated on 6% non-denaturing polyacrylamide gels that contained 0.1% bromophenol blue. Gels were dried and subjected to autoradiography. For competition assays, 30-fold excess unlabeled oligonucleotide was added (20 minutes, ambient temperature) after the above-described reaction with radiolabeled oligonucleotide had been carried out. Similarly, for super-shift assays, 2 μ g Ab was added (20 min, ambient temperature) after the reaction with radiolabeled oligonucleotide had been completed. Abs specific for p50 (sc-114X), p65 (sc-109X) or Myc (sc-764X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.7. Polymerase Chain Reaction (PCR) assays

Reverse transcription (RT) PCR and quantitative PCR (qPCR) relied on: total RNA extracted with the help of the TRIzol (Sigma-Aldrich, St. Louis, MO) reagent; cDNA synthesis using 1 μ g RNA and the AMV reverse transcriptase kit from Roche (Indianapolis, IN); and the gene-specific PCR primers listed in Supplemental Table 1. RT-PCR amplification of *LMP-1* (human herpesvirus 4), *Myc (c-myc)* and *Actb* (b-actin) message used the PCR master mix from Roche and 20–40 cycles of DNA amplification under the following cycling conditions: template denaturation at 95 °C for 5 minutes, primer annealing at 57 °C for 1 minute, and primer extension at 72 °C for 1 minute. 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 sec). 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. Probe sequences (5' to 3') were as follows: *E2F1*, AGG-GTC-TGC-AAT-GCT-ACG-AAG-GTC; *Myb*, TTA-TAG-TGT-CTC-TGA-ATG-GCT-GCG-GC; *Gadd45b*, ACG-AGC-TCT-TCC-AGC-GTC-ATG-TT; *Hprt*, AGG-ACT-GAA-CGT-CTT-GCT-CGA-GAT-G. 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 peripheral blood B cells and presented as fold relative expression (RE) change.

3. Results

3.1. PL inhibits proliferation of BL cells

To determine whether PL inhibits growth and proliferation of BL cells *in vitro*, we treated two EBV⁺ cell lines, Daudi and Raji, and two EBV⁻ cell lines, DG-75 and Ramos, for 24 hrs or 48 hrs with increasing concentrations of PL that ranged from 2.5 μ M to 20 μ M. MTS assay was employed to measure PL-dependent growth inhibition and estimate the concentration of compound at which cell growth was cut in half: IC₅₀. Figure 1B shows that the four BL lines included here varied in their susceptibility to PL. The IC₅₀ values after 24-hr exposure to compound ranged from 2.8 μ M in case of Daudi to 8.5 μ M in case of DG-75, a ~3-fold difference. In most studies described in the following, we used PL at IC₅₀, which was specific for each cell line. This permitted us to evaluate the effects of PL at equitoxic concentration levels. The result presented in Figure 1B demonstrated that low micromolar amounts of PL (<10 μ M) halt the growth of BL cells in a concentration- and time-dependent manner.

3.2. PL activates caspase-3 and causes apoptosis

Next we asked the question whether PL's ability to kill tumor cells selectively [19] may also be true for the B-cell lineage. BL and normal B220⁺ peripheral blood B-lymphocytes were treated for 24 hrs with PL (2.5–20 μ M) and the proportion of viable cells was determined using the TBE assay. Figure 2A shows that PL effectively killed BL cells in a concentration-dependent manner, whereas normal B cells remained largely unaffected. To evaluate whether PL-dependent killing of BL cells was caused by programmed cell death, we determined the fragmentation of genomic DNA and activation of caspase-3 by proteolytic cleavage – two widely recognized hallmarks of apoptosis. Figure 2B shows that treatment of BL with PL at IC₅₀ levels caused the nucleosomal DNA ladder that is typical for apoptotic cell death. Figure 2C shows that in all cell lines included in this study, treatment with PL activated caspase-3, generating a truncated protein with an apparent molecular weight of 19 kDa in case of DG-75, Daudi and Ramos and 17 kDa in case of Raji. To evaluate whether PL-mediated killing of BL cells can be exacerbated by agents that increase oxidative stress by elevating cellular levels of reactive oxygen species (ROS), we depleted PL-treated BL cells (24 hrs at IC₅₀) of the crucial antioxidant, glutathione (GSH), using co-treatment with the γ -glutamylcysteine synthetase inhibitor, buthionine sulfoximine (BSO; 1 mM). Supplemental Figure 1 shows that BSO significantly augmented PL-dependent cell death, whereas treatment with BSO on its own (without PL) was inconsequential. In contrast, co-treatment of BL cells with PL and the GSH-preserving antioxidant, dithiothreitol (DTT; 200 μ M), abrogated PL-dependent cell death. These results strongly implicate elevated oxidative stress in the mechanism by which PL causes apoptosis in malignant but not normal B-lymphocytes.

3.3. PL represses constitutive NF- κ B activity

Because NF- κ B activation is important for proliferation and survival of normal and neoplastic B-lymphocytes, we evaluated NF- κ B DNA-binding activity in BL cells using EMSA. Compared to normal B cells used as control (Fig. 3A, lane 1), all tumor cell lines included here exhibited grossly elevated NF- κ B DNA-binding activity (Fig. 3, lanes 2–5). To ascertain the specificity and subunit composition of NF- κ B, we conducted competition and super-shift assays using NE from BL cells. The results, which were the same for all 4 cell lines, are presented in Figure 3B and 3C using Daudi as example. Thus, incubation of NE with 30-fold excess unlabeled “competitor” probe (competes with labeled probe for NF- κ B dimers) abolished NF- κ B activity (Fig. 3B, lane 2), whereas incubation with a similar excess of unlabeled “mutant” probe (unable to bind NF- κ B dimers) had no effect (Fig. 3B, lane 3). This result indicated that the DNA-binding activity labeled “NF- κ B” in Figure 3A is in fact NF- κ B.

Because the heterodimer that consists of NF- κ B subunits, p50 and p65, is one of the most abundant forms of NF- κ B/Rel proteins in B cells [20], we performed a super-shift assay with Ab to p50 or p65 to determine whether BL cells harbor heterodimers of this sort. Figure 3C shows that compared to an untreated control sample (lane 1), the addition of Ab to p50 (lane 2) and p65 (lane 3) shifted NF- κ B activity to higher molecular weight locations in the gel (indicated by red arrowheads). Ab to Myc, used as a control for the specificity of the reaction, was ineffective (lane 4). The finding that Ab to p50 super-shifted two distinct NF- κ B fragments, but Ab to p65 super-shifted only the upper fragment, suggested that NF- κ B activity in BL cells is mainly comprised of p50/p50 homodimers (lower super-shifted fragment in lane 2) and p50/p65 heterodimers (upper super-shifted fragment in lanes 2 and 3). Supplemental Figure 2 presents similar results for DG-75, Raji and Ramos cells.

To investigate whether PL inhibits NF- κ B in BL, we cultured the tumor cells in presence of drug at IC₅₀ levels. Treatment for 24 hrs dramatically inhibited NF- κ B activity in all 4 cell

lines (Fig. 3D). Because PL's inhibitory effect on NF- κ B could be caused by blocked degradation of I κ B α , we used Western blotting to examine whether treatment of tumor cells with PL results in accumulation of the protein in the cytosol. The results, which were the same for all 4 cell lines, are presented in Figure 3E using Daudi as example. The increase in I κ B α (top panel) was accompanied by an elevation of p65 in the cytosol (2nd panel) and a concomitant reduction of p65 in the nucleus (3rd panel). Our previous work with NF- κ B inhibitors, curcumin and arsenic trioxide, had shown that these compounds suppress NF- κ B, in part, by interrupting the binding of NF- κ B dimers to DNA target sites [21; 22]. To evaluate whether this may also be true for PL, we added PL to NE from Daudi and NF- κ B binding probe *prior* to running EMSA. Figure 3F shows that PL somewhat reduced the NF- κ B activity, particularly the upper portion of the activity that corresponds to p50/p65 heterodimers (indicated by red arrowhead in lane 2). Supplemental Figure 3 presents similar results for DG-75, Raji and Ramos cells.

These results suggested that PL diminishes NF- κ B activation by a complex mechanism that involves reduced degradation of I κ B α , decreased nuclear translocation of p65, and diminished binding of p50/p65 to cognate DNA sequences.

3.4. PL down regulates MYC DNA-binding activity

Deregulated expression of *MYC* (*c-myc*) due to reciprocal chromosomal translocation that juxtaposes the cellular oncogene to immunoglobulin gene enhancers is a long-standing hallmark of BL [23; 24]. Because our previous work with iMyc^{E μ -1} cells, a mouse model of the human *BLMYC*-activating t(8;14) translocation, had shown that cancer drug-induced inhibition of mouse *Myc* can lead to dramatic reduction in cell growth and survival [25], we here decided to examine the DNA-binding activity of MYC in human BL cells. Figure 4A presents EMSA results indicating that MYC is highly activated in the four cell lines included in this study. Competition assays (Fig. 4B) and super-shift assays (Fig. 4C) confirmed the finding, using Daudi as example. Supplemental Figure 4 presents similar results for DG-75, Raji and Ramos. Treatment of BL cells with PL at IC₅₀ levels for 24 hrs abolished the MYC activity in DG-75 cells (Fig. 4D, lanes 1–2) and greatly reduced it in Daudi, Raji and Ramos cells (Fig. 4D, lanes 3–8). Unlike PL's apparent ability to interrupt the binding of NF- κ B to DNA, PL did not directly block DNA binding of MYC in Daudi cells (Fig. 4E). Supplemental Figure 5 shows that this was also true for DG-75, Raji and Ramos cells. These results suggested that inhibition of MYC is also involved in PL-dependent growth and survival inhibition of BL. In keeping with NF- κ B's known function as a direct regulator of MYC [26], it is likely that the observed abrogation of MYC is a consequence of suppressed NF- κ B activity, effected in part by NF- κ B-dependent reduction of *MYC* transcription. The significant drop in *Myc* mRNA levels seen in 4 of 4 PL-treated BL cell lines lent support to this possibility (Supplemental Figure 6).

3.5. PL changes expression of cellular NF- κ B/MYC target genes, E2F1, MYB and GADD45B

We determined the expression of 30 selected NF- κ B/MYC target genes to elucidate the mechanism by which PL-dependent inhibition of the NF- κ B/MYC pathway kills BL cells. qPCR analysis of total RNA obtained from BL and normal B cells that were either treated with PL (24 hrs, IC₅₀) or left untreated pointed to three genes that were consistently deregulated in all BL cell lines: *E2F1*, *MYB* and *GADD45B*. Figure 5A shows that expression of *E2F1* and *MYB* was reduced up to 3-fold and 7-fold, respectively. In contrast, expression of *GADD45B* was consistently up regulated, resulting in a moderate increase in Daudi and Ramos and a high increased in DG-75 and Raji. Somewhat unexpectedly, genes that encode pro-apoptotic proteins, such as BAX and BIM, or anti-apoptotic proteins, such as BCL2 and BCL-X_L, were not affected by PL (not shown). These results suggested that

PL inhibits BL by affecting the expression of a key subset of NF- κ B/MYC targets, including *E2F1*, *MYB* and *GADD45B*.

3.6. PL inhibits expression of EBV-encoded LMP-1 in Daudi and Raji cells

Epstein-Barr virus (EBV), which is associated with a broad spectrum of B-cell lymphomas including BL [27], encodes the oncoprotein, late membrane protein-1 (LMP-1), a known activator of NF- κ B signaling in B cells [28]. Because NF- κ B and MYC function as master transcriptional regulators in EBV-immortalized B-lymphocytes [29], and we observed that treatment of BL with PL suppresses NF- κ B and MYC activity, we hypothesized that PL-dependent killing of EBV⁺BL may be accompanied by down regulation of *LMP-1* at the transcriptional level. To follow up on this possibility, we employed RT-PCR to demonstrate that *LMP-1* is expressed in Daudi and Raji cells. Figure 5B shows that this was the case, using normal B cells as control. Next we examined whether treatment with PL might quench *LMP-1*. Daudi and Raji cells treated for 24 hrs with PL at IC₅₀ harbored levels of *LMP-1* message that were greatly reduced (Fig. 5C). EBV⁻DG-75 was included as control. It is possibly that the PL-dependent drop in NF- κ B activity in EBV⁺BL is caused, in part, by diminished *LMP-1* expression, but this has not been shown here.

4. Discussion

This report extends the main finding of a recent study on solid cancer [19] to the liquid cancer, BL. The results presented here demonstrate that PL inhibits BL in a concentration- and time-dependent manner: the IC₅₀ after 24-hr treatment with compound ranged from 2.8 μ M (Daudi) to 8.5 μ M (DG-75) – similar to the concentration levels of PL that killed fifty percent of leukemia cells of the myeloid (HL-60, K562) or T-cell (Jukart, Molt-4) lineage in studies by other investigators [30]. It has been postulated that PL kills carcinoma cells by targeting their “non-oncogene co-dependency” on elevated anti-oxidative defense pathways acquired in response to cell transformation-induced oxidative stress [19]. Besides GSTP1 and CBR1, this co-dependency may render glyoxalase I (GLO1), peroxiredoxin 1 (PRDX1) and 3 members of the glutathione transferase family of proteins (GSTZ1, GSTM3, GSTO1) molecular targets of PL in carcinoma cells [19]. Additional studies are warranted to assess whether the anti-oxidative defense is also a key target of PL in malignant B-lineage including BL cells. PL-dependent genotoxicity [31] and inhibition of hypoxia inducible factor-2 (EC₅₀ 4.8 μ M) [32] may also be involved in the mechanism by which the compound kills tumor cells. Our findings in BL have added reduced NF- κ B/MYC signaling and changed expression of key target genes (*E2F1*, *MYB*, *GADD45B*, *LMP-1*) to that mechanism.

Our observation that NF- κ B is constitutively active in BL cells, and that PL inhibits NF- κ B activity in these cells by blocking both the degradation of I κ B α and the translocation of p50/p65 to the nucleus, is in line with the widely accepted paradigm that NF- κ B signaling plays a critical role in B-lineage tumors and, therefore, provides an attractive target for intervention strategies [33]. Using TNF- α -induced NF- κ B activation as experimental readout, Sing *et al.* were first to show that PL inhibits NF- κ B [34], although this study did not include B cells. Our previous work two phytochemicals that are closely related to PL, capsaicin and curcumin, also drew attention to NF- κ B. Capsaicin suppressed phorbol ester-induced NF- κ B activation in mouse epidermis and human promyelocytic leukemia cells by virtue of inhibiting I κ B α degradation [21; 35], whereas, in marked analogy to the findings reported here, curcumin caused growth arrest and apoptosis of B-lymphoma cells by suppressing NF- κ B, stabilizing I κ B α , and interrupting NF- κ B binding to DNA [36]. It is possible that in the BL cell lines included here, PL-induced suppression of NF- κ B led to down regulation of MYC activity. *MYC* is a known transcriptional target of NF- κ B, and NF- κ B dependent abrogation of mouse *Myc* message and Myc protein resulted in a newly

developed mouse model of human BL in a dramatic decrease in proliferation and survival [37]. Down regulation of Myc was also seen in CDDO-Im induced killing in the same mouse model [25] and curcumin-induced killing of an unrelated model of B-lymphoma [38]. In aggregate, these findings support the contention that NF- κ B-dependent suppression of MYC contributes to the mechanism by which PL kills BL cells.

NF- κ B/MYC-regulated gene expression networks in B-lineage cells, which are known to govern hundreds if not thousands of target genes, have been elucidated in great depth and deposited in public databases [39]. We took advantage of that and used qPCR analysis to screen a candidate list of validated NF- κ B/MYC target genes (n=30) with regard to PL-induced expression change. The result led us to focus on 4 genes that were consistently deregulated in PL-treated BL cells: *E2F1*, *MYB* and *LMP-1* were suppressed, whereas *GADD45B* was activated. *E2F1*, a direct target gene of MYC (www.myc-cancer-gene.org), encodes a transcription factor important for NF- κ B-dependent survival of tumor cells [40], cancer progression [41] and resistance of cancer cells to chemotherapy [41]. *MYB*, a direct target gene of NF- κ B [20], encodes a transcriptional activator whose abrogation in cancer cells causes apoptosis [42]. *LMP-1*, arguably the most important oncogene in the EBV genome [43], orchestrates a Rel/NF- κ B/MYC-centered “master” transcription program that is crucial for immortalization and malignant transformation of B-lymphocytes [29]. Down regulation of the 3 genes mentioned above would be expected to promote cell death, as seen in our study on PL-treated BL. The reason for the observed PL-dependent upregulation of *GADD45B* is less clear because NF- κ B has been reported to induce the gene via binding to three proximal κ B elements in the promoter region [44]. Nonetheless, induction of *GADD45B* upon treatment with PL is in line with emerging evidence that the gene is a sensor of cellular stress responses that can govern the switch from cell survival to programmed cell death [45], as recently shown for Fas-mediated apoptosis [46].

5. Conclusions

Although additional studies are required before the mechanism of PL-induced cell killing can be fully appreciated, the results reported here lend credence to proposals to include PL in therapeutic regimens of malignant B-cell tumors [47] and consider it for new approaches to the chemoprevention and treatment of other types of blood cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

| | |
|--------------------|-----------------------------|
| Ab(s) | Antibody(ies) |
| BL | Burkitt lymphoma |
| CE | Cytoplasmic protein extract |
| <i>E2F1</i> | E2F transcription factor 1 |
| EBV | Epstein Barr virus |

| | |
|--------------------------------|---|
| EMSA | Electrophoretic mobility shift assay |
| GADD45B | Growth arrest and DNA-damage-inducible 45 beta |
| LMP-1 | Latent membrane protein 1 (human herpesvirus 4 a.k.a. Epstein Barr virus) |
| MYB | Myeloblastosis oncogene |
| MYC | Myelocytomatosis oncogene |
| NE | Nuclear protein extract |
| NF-κB | Nuclear factor kappa-B |
| PL | Piperlongumine |
| ROS | Reactive oxygen species |
| TBE | Trypan blue exclusion |
| TE | Total cell protein extract |

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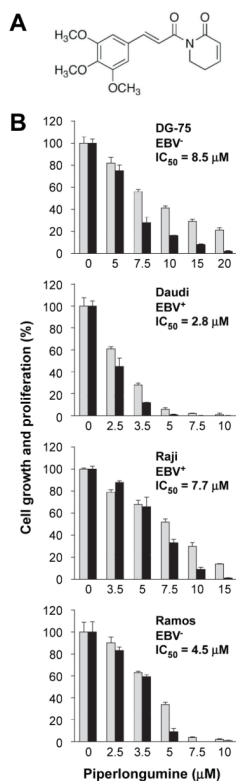


Figure 1. PL inhibits proliferation of BL cells in a time- and concentration-dependent manner (A) Chemical structure of piperlongumine (PL).

(B) PL-dependent growth inhibition of BL. Tumor cells were seeded in 96-well plates at a density of 1×10^6 /ml and grown in presence of the indicated concentration of PL, either for 24 hrs (grey bars) or 48 hrs (black bars). Cell proliferation was measured using the MTS assay. Error bars represent standard deviations of the mean determined in a representative experiment performed in triplicate. IC₅₀ is indicated for the 24-hr treatment. These conditions were used for all experiments presented in Figures 2–5 unless otherwise stated.

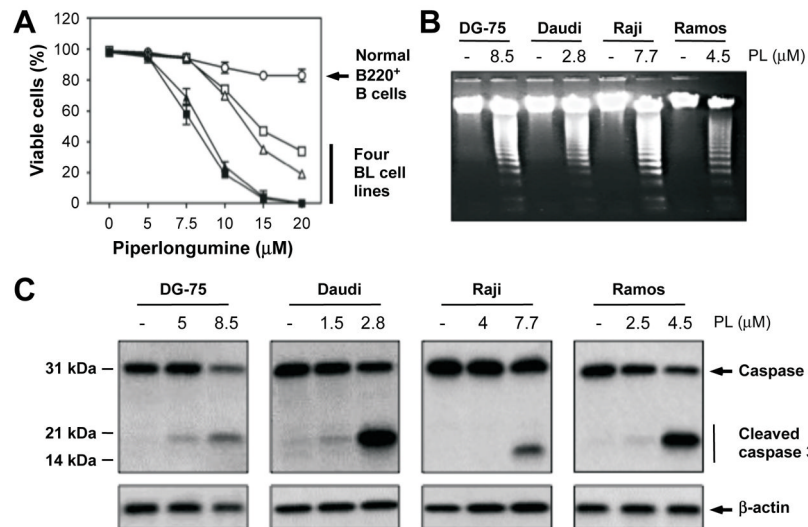


Figure 2. PL-induced apoptosis involves activation of caspase-3

(A) PL kills BL but not normal B cells. Normal peripheral blood B-lymphocytes obtained by MACS separation using B220 columns (circles) and BL cells (open squares, DG-75; closed squares, Daudi; open triangles, Raji; closed triangles, Ramos) were plated at a density of 1×10^6 /ml and treated with PL for 24 hours. Viable and dead cells were enumerated using the trypan blue exclusion (TBE) assay and a hemocytometer. Symbols represent mean values of percent viable cells based on a representative experiment performed in triplicate. Error bars indicate the standard deviation of the mean.

(B) Treatment with PL leads to DNA fragmentation. BL cells were treated as described above and genomic DNA was fractionated by agarose gel electrophoresis and stained with ethidium bromide. DNA fragmentation was not observed in normal B-lymphocytes treated with 8.5 μM PL (not shown).

(C) Treatment with PL activates the executioner caspase, caspase-3, by cleaving the proenzyme. BL cells were treated as described in panel A. Total cell lysate was prepared and subjected to Western analysis using Ab that detects both pro-caspase-3 and the active, cleaved form of the protein. Ab to β-actin was used as loading control. Caspase activation was not seen in normal B-lymphocytes treated with 8.5 μM PL (not shown).

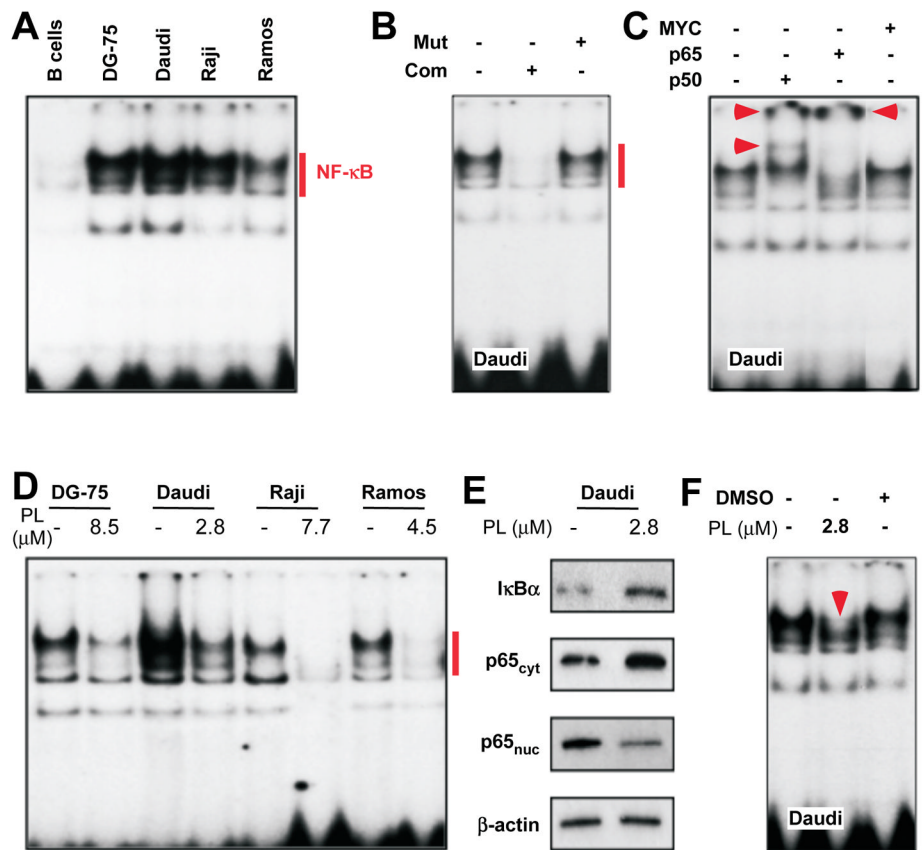


Figure 3. Treatment with PL results in reduced NF- κ B activity

(A) BL cells harbor constitutive NF- κ B activity. EMSA used 10 μ g NE prepared from normal B cells (lane 1) or BL cells (lanes 2–5). NF- κ B activity is indicated by a red vertical line that is labeled.

(B) Confirmation of NF- κ B DNA binding using competition assay. NE (10 μ g) was incubated with excess unlabeled “competitor” oligonucleotide probe (lane 2) or excess unlabeled “mutant” probe (lane 3) as described in Materials and Methods.

(C) Determination of NF- κ B dimers by virtue of super-shifts. NE (10 μ g) was incubated with 2 μ g of one of the following Abs: p50 (lane 2), p65 (lane 3) or Myc (lane 4). Red arrowheads denote shifted bands.

(D) PL decreases NF- κ B levels. NE was prepared from BL cells treated with PL for 24 hrs or left untreated. EMSA was used to determine the drop in NF- κ B activity.

(E) Treatment with PL results in accumulation of I κ B α and p65 in the cytosol. NE and CE were prepared from Daudi cells treated with PL for 24 hrs. Untreated cells were used as control. I κ B α and p65 were detected by Western blotting. β -actin was used as loading control.

(F) PL partially blocks binding of NF- κ B to DNA *in vitro*. PL was dissolved in DMSO and added to NE from Daudi cells; 20 min later NF- κ B activity was determined (lane 2). NE treated with DMSO (solvent control, lane 3) or left untreated (lane 1) was used for comparison.

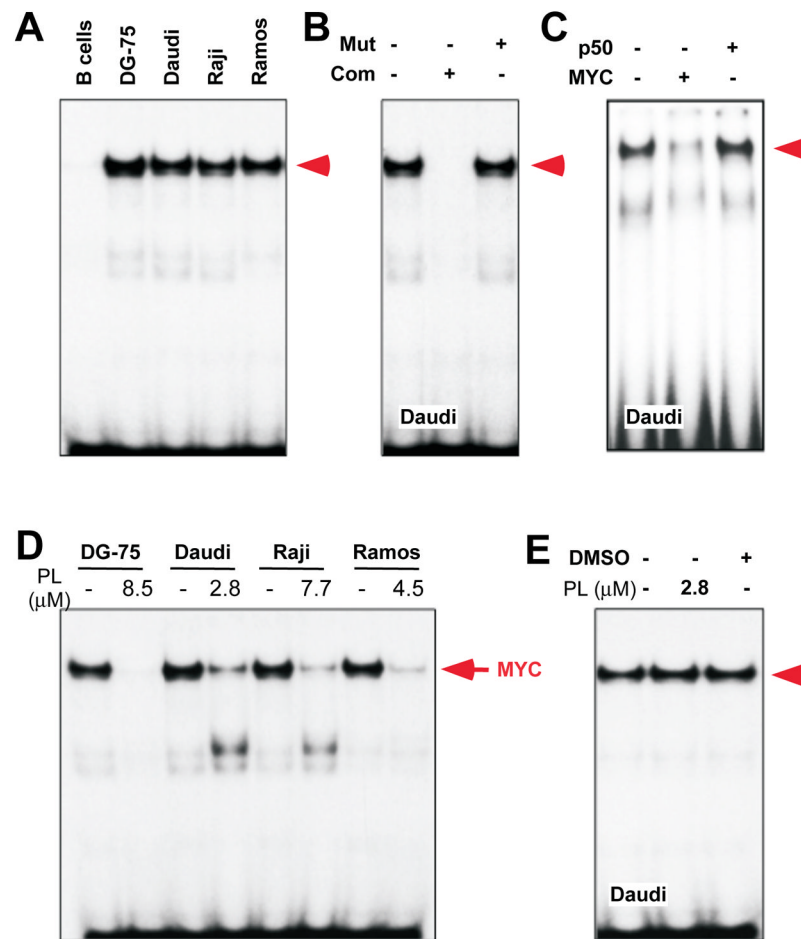


Figure 4. Treatment with PL blunts MYC DNA-binding activity

(A) BL cells contain constitutive MYC activity. MYC DNA-binding activity (indicated by red arrowhead) was determined by EMSA using 10 μg NE.

(B) Confirmation of MYC DNA-binding activity using competition assays. NE (10 μg) was incubated with either excess unlabeled “competitor” probe containing cognate MYC sequence (lane 2) or excess unlabeled “mutant” probe containing an unrelated sequence (lane 3).

(C) Confirmation of MYC DNA-binding activity using supershift assay. NE (10 μg) was left untreated (lane 1) or incubated with Ab (2 μg) to MYC (lane 2) or p50 (lane 3) prior to EMSA. The image is representative of three experiments that yielded the same result.

(D) PL-dependent drop in MYC DNA-binding activity. NE was prepared from BL cells treated with indicated amounts of PL for 24 hrs or left untreated. EMSA was used to determine levels of MYC activity.

(E) PL does not interfere with binding of MYC to DNA *in vitro*. NE from Daudi cells was treated with PL (lane 2) or DMSO (lane 3) or left untreated (lane 1). Twenty minutes later EMSA was used to determine MYC binding to a labeled, MYC-specific oligonucleotide probe.

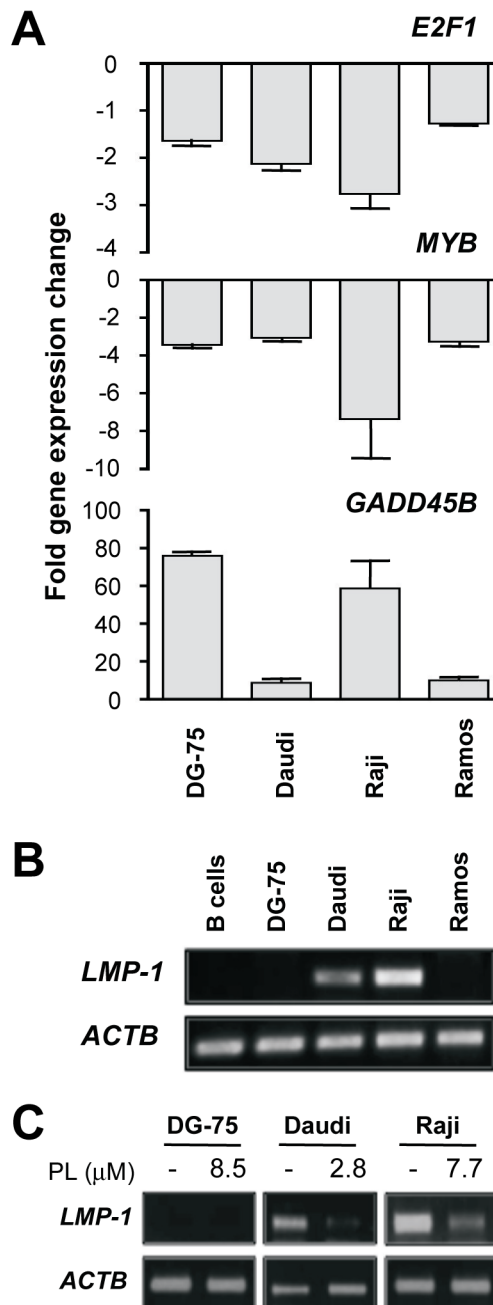


Figure 5. Treatment with PL changes expression of cellular NF- κ B/MYC target genes and the EBV-encoded oncogene, *LMP-1*

(A) BL cells treated with PL at IC_{50} for 24 hrs exhibit reduced expression of *E2F1* and *MYB* and increased expression of *GADD45B*. Total RNA was analyzed using qPCR. Gene expression levels were normalized to levels of *HPRT* message and expressed as fold change in PL-treated cells versus untreated cells. Mean values and standard deviations of an experiment performed in triplicate are presented as columns and error bars, respectively.

(B) RT-PCR detects *LMP-1* mRNA in EBV⁺ Daudi and Raji cells but not in EBV⁻ BL (DG-75 and Ramos) or normal peripheral blood B cells.

(C) PL inhibits *LMP-1* expression in Daudi and Raji cells. DG-75 was included as control. BL cells (1×10^6 /ml) were treated with PL at IC_{50} for 24 hrs. Total RNA was prepared and analyzed using RT-PCR.

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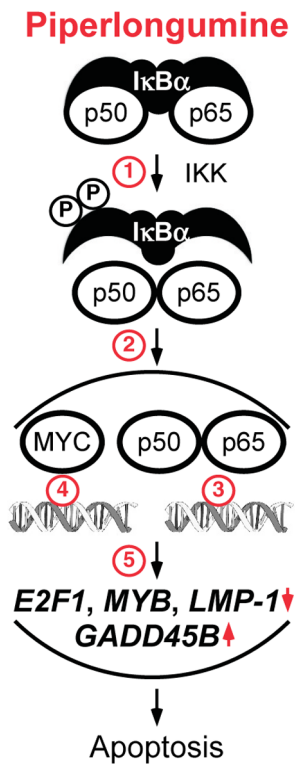


Figure 6. Scheme of proposed mechanism by which PL inhibits BL

This study has shown that PL inhibits NF- κ B signaling by blocking the degradation of I κ B α (indicated by 1; cf. Fig. 3E top), curbing the translocation of p65 from the cytoplasm to the nucleus (2; Fig. 3E, panels 2 and 3), and interfering with the binding of NF- κ B dimers to target sites in gene promoters (3; Fig. 3F). Treatment with PL also leads to reduced MYC DNA-binding activity (4; Fig. 4D), presumably by a mechanism that involves NF- κ B-dependent down regulation of MYC. Diminished NF- κ B/MYC activity results in deregulated expression of the cellular NF- κ B/MYC target genes, *E2F1*, *MYB* and *GADD45B* (Fig. 5A), and, in cells harboring EBV, the virus-encoded gene, *LMP-1* (5; Fig. 5C). The net effect of these changes is programmed cell death.