

Antileukemic effects of piperlongumine and alpha lipoic acid combination on Jurkat, MEC1 and NB4 cells *in vitro*

ABSTRACT

Aim of Study: This research indicated to evaluate the effects of piperlongumine (PL), a biologically active alkaloid, and alpha lipoic acid (ALA), a naturally occurring cofactor existed in multienzyme complexes regulating metabolism on leukemia cells. Excessive production of reactive oxygen species (ROS) can lead to oxidative stress, a state that has been observed in several hematopoietic malignancies, including acute and chronic myeloid leukemias. The importance of the association between oxidative stress and malignancy is not currently clear; however, there is evidence that tumor-derived ROS may promote cell survival, migration and metastasis, proliferation and even drug-resistance depending on the origin of the cancer. Increased oxidative stress in leukemic cells may represent a potential therapeutic target, although there are differing opinions on whether therapeutic strategies should aim to antagonize or further promote oxidative stress in leukemic cells.

Materials and Methods: The effects of PL alone (5, 15, 30 μ M) and in combination (30 μ M) with ALA (200 μ M) on Jurkat, NB4 and MEC1 leukemia cell lines were investigated through MTT, caspase-3 and cyclooxygenase-2 (COX-2) activities.

Results: Inhibition of COX-2 and the induction of caspase-3 cleavage in Nb4 (acute promyelocytic leukemia) cells were found to be significant following PL application and synergistic effects with combination of ALA (inhibition of COX-2 as 23.74% and 3.55-fold increase of caspase-3).

Conclusion: PL and ALA may have a potential value as a therapeutic agent for patients with acute promyelocytic leukemia.

KEY WORDS: Alpha lipoic acid, COX-2, leukemic cell lines, oxidative stress, piperlongumine

INTRODUCTION

Cancer is one of the major public health problems, which figures among the leading causes of death worldwide. Among the estimated new cancer cases in United States for 2014, leukemia (acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, chronic myeloid leukemia and other leukemia) is expected to comprise around 3%.^[1] Leukemia is a heterogeneous hematological malignancy that starts in blood-forming tissue, such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the bloodstream.^[2] Along with conventional treatment methods, medicinal plants and their phytoconstituents have always been accepted as complementary and alternative choice for the treatment of leukemia, and nutraceuticals have been proved to have antileukemic activity in experimental studies.^[3,4]

Piperlongumine (Piplartine, 5,6-dihydro -1-[(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2propenyl] -2(1H)-pyridinone), biologically active alkaloid/amide component from piper species (*Piperaceae*), which

are used in ethnopharmacology for treating tumors, diseases of the spleen, malaria, viral hepatitis, bronchitis, cough, asthma, respiratory infections, stomachache, and gonorrhea in Asia and Pacific islands especially in Indian medicine through several biological mechanisms such as antiplatelet aggregation, antinociceptive, anxiolytic, antidepressant, antiatherosclerotic, antidiabetic, antibacterial, antifungal, leishmanicidal, trypanocidal, schistosomicidal activities.^[5,6] This naturally occurring small molecule recently identified to be toxic selectively to cancer cells *in vitro* and *in vivo* through elevation of the cellular levels of reactive oxygen species (ROS) and cellular cross-linking events like irreversible protein glutathionylation mechanisms.^[7] Anticancer properties were previously investigated through cytotoxic effects in several tumor cell lines (colon, lung, prostate, embryonal carcinoma, glioblastoma, neuroblastoma, melanoma, breast carcinoma, etc.), genotoxicity, antitumor effects, angiogenic and antimetastatic mechanisms.^[5]

Despite the numerous literatures available for the cytotoxic antitumor effects of piperlongumine, still

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better understanding for the mechanism of action is required. Therefore in the current study, the effects of piperlongumine on three different leukemia cell lines: Jurkat, NB4 and MEC1 were investigated through MTT, caspase-3 and cyclooxygenase-2 (COX-2) activity. NB4 was derived from the marrow of a patient with acute promyelocytic leukemia (APL) in relapse^[8] with a specific chromosome translocation on t (15;17) (q22;q11-12) characterized in APL, which is a subtype of acute myeloid leukemia.^[9] MEC1 cells are derived from the peripheral blood of a Epstein-Barr virus (EBV)-positive 58-year-old Caucasian male patient with B-chronic lymphocytic leukemia (B-CLL) in prolymphocytoid transformation^[10] representing the pre-aggressive clinical stage from an EBV carrying cell within the subclone that was in the early prolymphocytic transformation stage^[11] where despite negative CD5 expression, is accepted as a good model for B-CLL.^[12] Jurkat, the immortalized IL-2-independent human T-lymphocyte cell line, is used to study acute T-cell leukemia.^[13] Alpha lipoic acid (ALA), a naturally occurring cofactor existed in multienzyme complexes regulating metabolism were found to induce apoptosis in various cancer cell lines.^[14] As a potent anticancer agent, which binds to the active sites of several key cellular antioxidants, including glutathione S-transferase and carbonyl reductase 1, piperlongumine could not raise ROS amounts in normal cells because of the lower quantity of antioxidants due to less activity of Nrf2 (NF-E2-related factor 2) transcription factor.^[15] Therefore, with the knowledge of anticancer properties, the current study was aimed to study the effects of piperlongumine in selected leukemia cell lines and its combination with ALA.

MATERIALS AND METHODS

Cell culture and treatments

Jurkat cells and NB4 cells were provided by Prof. Taner Ogurtas from Gulhane Medical School and MEC1 cells were provided by Prof. Serap Erdem from Istanbul University, Faculty of Medicine. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Fetal Bovine Serum, Irvine Scientific, Santa Ana, California, USA), penicillin (100 IU/mL) and streptomycin (100 µg/mL), 2 mM L-glutamine, (GibcoBRL, USA). Cells were maintained in a 37°C, 5% CO₂, fully humidified incubator and subcultured every 3–4 days. Cells were treated at 5, 10 and 15 µM concentrations of piperlongumine alone and in combination at 30 µM concentration with ALA (200 µM) along with DMSO (dimethyl sulfoxide) vehicle control wells and harvested after 24 hours. For this, cells were lysed by cell lysis buffer (RIPA Buffer, Sigma-Aldrich, cat. R0278) on ice for 10 min, and then centrifuged at 10,000 g for 1 min. Protein concentrations of the treated cells were determined using BCA Protein Assay Reagent (Pierce, Rockford, IL). Protein concentrations in the cell lysates were adjusted to 0.3 mg/mL for COX-2, and 1 mg/mL for caspase-3 assays.

Caspase-3 and COX-2 ELISA assays

Quantification of cleaved caspase-3 activity was performed using the PathScan Cleaved Caspase-3 (Asp175) sandwich

ELISA kit (no. 7190, Cell Signaling Technology, USA) and total COX-2 activity was conducted using PathScan Total COX-2 sandwich ELISA kit (no. 7291, Cell Signaling Technology, USA) according to the manufacturer's recommendations. Caspase-3 assay kit detects endogenous levels of cleaved caspase-3 protein and COX-2 kit detects the endogenous levels of COX-2. For this purpose, cells were plated in 12-well plates and cultured for 24 h. Plates were washed with PBS and treated with piperlongumine at 5, 15 and 30 µM concentrations along with alanine (200 µM) alone and alanine (200 µM) + piperlongumine (30 µM) combination. After incubation, cells were washed twice with cold PBS and 100 µl lysis buffer was added. Lysates were centrifuged and supernatant was used for ELISA for both assays.

MTT assay

Cytotoxicity in the cells were monitored using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Following 24 h cultivation of cells with piperlongumine, media was removed from the cells and 10 µL of MTT solution (0.5 mg/mL in the PBS) was added to each well. The plates were then incubated at 37°C for 3 h. Formazan formation was inspected under microscope and extracted by dissolving in 100 µL 1% SDS (sodium dodecyl sulfate). The cell viability was quantified using a microplate reader (Tecan, Sunrise, Switzerland) at 540 nm.^[16] A plot of % cytotoxicity versus sample concentrations was used to calculate the concentration, which showed 50% cytotoxicity (IC₅₀).

RESULTS

Cytotoxic activity

The effect of piperlongumine on leukemia cell lines at 2.5–30 µM concentrations were evaluated by MTT assay; where cell death of the APL cells (NB4) were found to have increased in a concentration-dependent manner. 2.5 µM piperlongumine induced no cytotoxic activity; however, at 15 and 30 µM doses, cell viability were found to have decreased by 73–78%. Calculated IC₅₀ for NB4 cells were found to be 8.97 µM. For Jurkat and Mec1, cell proliferation was observed at 2.62–15.49%, independent from the increasing concentrations [Figure 1].

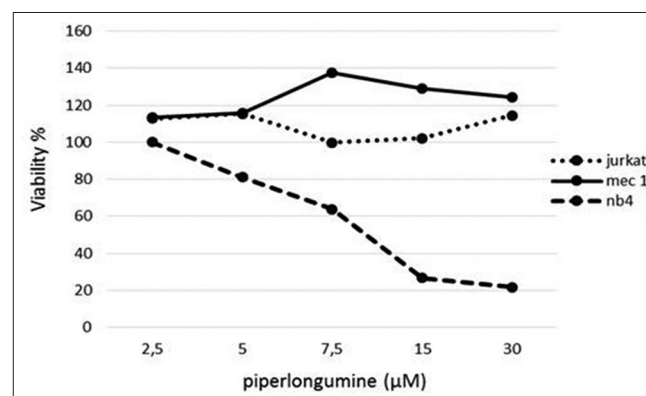


Figure 1: Cell viability (%) of piperlongumine (2.5–30 µM) on Jurkat, MEC1, NB4 cell lines by MTT assay (24hr incubation)

Caspase-3 cleavage

At the end of 24-hour incubation period, while no significant change was observed for Jurkat cells, at 5, 15 and 30 μM concentrations, caspase-3 cleavage was found to be increased 1.77, 1.85 and 1.91 folds for Mec1 cells. This apoptotic induction was found to be higher in NB4 cells, as 1.38-, 2.60- and 4.63-fold increase for the mentioned doses respectively, in accordance to the cytotoxicity results. On the contrary, when cells were treated by ALA at 200 μM and in combination with piperlongumine at 30 μM ; caspase-3 cleavage were found to have decreased in Jurkat cells (1.33- and 1.36-fold decrease), and increased in NB4 cells (2.82- and 3.55-fold increase) ($P < 0.05$); where no change was observed for Mec1 cells [Figures 2 and 3].

COX-2

The level of COX-2 did not change with the increased concentrations of piperlongumine, while a significant decrease

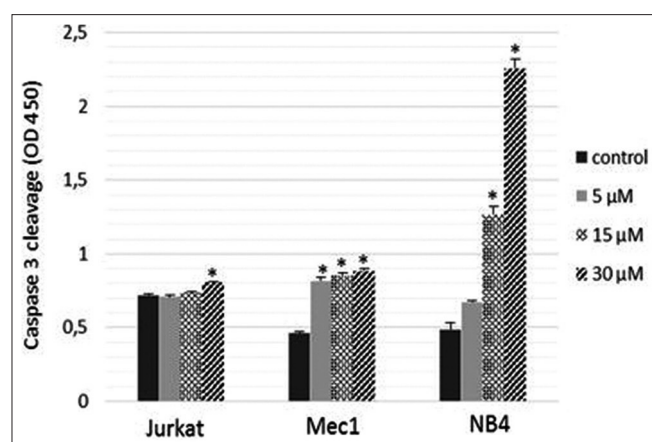


Figure 2: Effect of piperlongumine alone (5, 15, 30 μM) on the caspase-3 cleavage in Jurkat, Mec1 and NB4 cells. The data are expressed as the mean $\pm$ standard deviation of 3 experiments, each conducted in quadruplicate (* indicate the values were significantly different than the controls, $P < 0.05$)

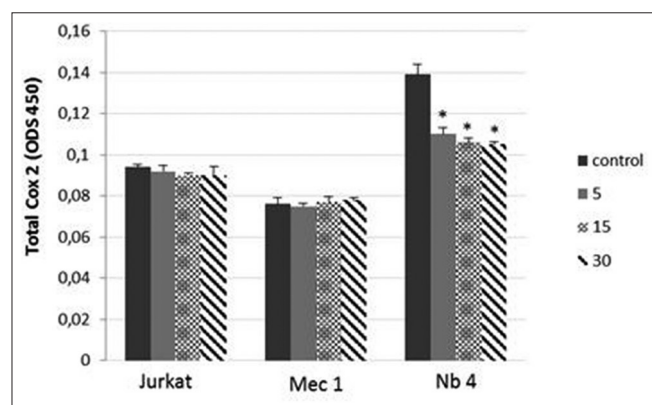


Figure 4: Effect of piperlongumine alone (5, 15, 30 μM) on the endogenous COX-2 in Jurkat, Mec1 and NB4 cells. The data are expressed as the mean $\pm$ standard deviation of 3 experiments, each conducted in quadruplicate (* indicate the values were significantly different than the controls, $P < 0.05$)

was observed for NB4 cells compared to control groups ($P < 0.05$) by increased doses (20.86%, 23.74%, 24.46%). ALA and piperlongumine combination increased the COX-2 levels for Jurkat (9.57%) and Mec1 cells (36.84%); while a decrease (23.74%) was observed for NB4 cells. ALA alone at 200 μM also increased the levels of COX-2 in Mec 1 cells (19.94%) [Figures 4 and 5].

DISCUSSION

Piperlongumine, a compound isolated from peppers, which are widely used in Ayurvedic medicine, showed to be a promising anticancer agent through several properties such as induction of oxidative stress selectively in cancer cells and genotoxicity supported by its weak systemic toxicity and safety for long-term consumption.^[5]

The antioxidant, sulfur-containing fatty acid, ALA has been shown to induce apoptosis and affect the biological processes

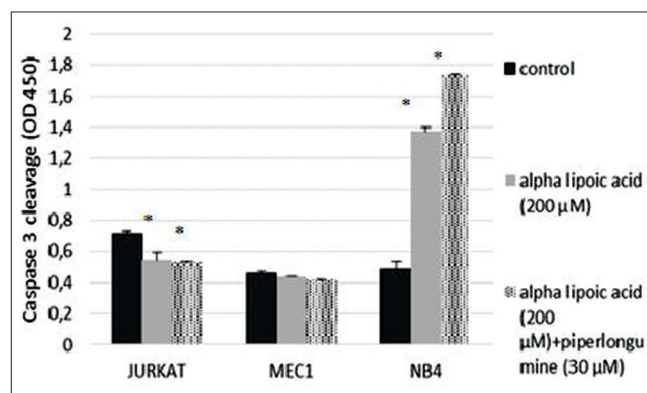


Figure 3: Effect of alpha-lipoic acid (200 μM) alone and in combination with piperlongumine (30 μM) on the caspase-3 cleavage in Jurkat, Mec1 and NB4 cells. Data are expressed as the mean $\pm$ standard deviation of three experiments, each conducted in quadruplicate (* indicate the values were significantly different than the controls, $P < 0.05$)

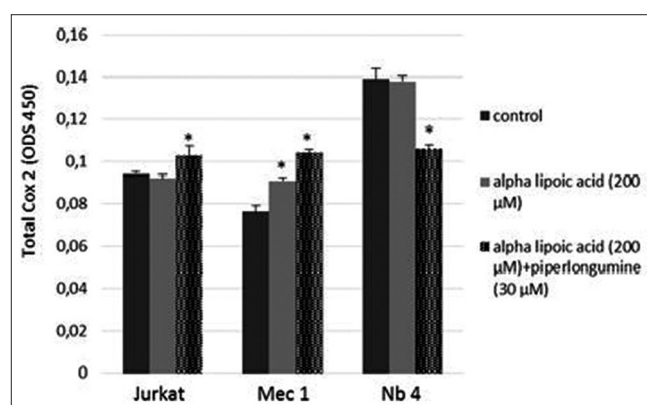


Figure 5: Effect of alpha-lipoic acid (200 μM) alone and in combination with piperlongumine (30 μM) on the endogenous COX-2 in Jurkat, Mec1 and NB4 cells. Data are expressed as the mean $\pm$ standard deviation of 3 experiments, each conducted in quadruplicate (* indicate the values were significantly different than the controls, $P < 0.05$)

including cancer via different pathways.^[17,18] ALA-induced apoptosis was dependant on the activation of the caspase cascade (increased caspase-9 and caspase-3 activity) and the mitochondrial death pathway.^[18] Pack *et al.*^[19] studied the effects of ALA on leukemic T cell lines; Jurkat and human lymphoblasts (CCRF-CEM) and normal human peripheral blood lymphocytes at 0.1, 1 and 10 μM concentrations. ALA was found to be cytotoxic for both leukemic cell lines (IC50 for inhibition of DNA synthesis were found as $1.6 \pm 0.9 \mu\text{M}$ in Jurkat cells and $2.6 \pm 0.7 \text{ mM}$ for CCRF-CEM cells) and this selective cytotoxic effect was found to be induced through apoptotic pathways shown by electron microscopy. Sen *et al.*^[20] showed that treatment of Jurkat cells with 100 μM ALA for 72 h potentiated Fas-mediated apoptosis through caspase-3 activated pathway. 200-800 μM ALA application on HL-60 promyelocytic leukemia cells were found to affect the level of ATP production and mediated apoptosis causing toxicity; where on blood lymphocytes, it was found to be non-toxic.^[21] In the current study, increased caspase-3 levels were observed only for acute promyelocytic Nb4 cells like HL-60 cells and not for Jurkat and Mec1, which was more evident with piperlongumine combination treatment.

Effects of ALA on inflammation with the mediation of COX-2 during several pathological conditions were studied previously^[6,22] and a link between the modulation of stromal inflammation via inhibition of COX-2 expression were found for leukemic cells,^[23] which is important for tumor progression through cancer-related inflammation, tumor invasion, resistance to apoptosis and suppression of antitumor immunity.^[24] Epigenetic gene silencing is a common cancer-associated mechanism resulting in dramatic transcriptional repression, most notably of tumor suppressor genes, during tumorigenesis.^[25] However, the mechanism is complex and the inhibition of proliferation and the induction of apoptosis in leukemia cells (K562, NB4, U937, HL60, and CEM cells) could also be via COX-2 independent pathways even by the administration of selective COX-2 inhibitor, Etadolac.^[26] Shams *et al.*^[27] demonstrated the negative correlation between the COX-2 and caspase-3 expression in the tissues of colon cancer patients and related these results to the inhibitory effect of COX-2 on the apoptotic process. Similarly, in the current study, COX-2 levels in the treated cells were found to present negative correlation; especially for Nb4 cells. Also previously, the induction of caspase-mediated apoptosis through caspase-3 activation in PC-3 human prostate cancer cells by piperlongumine at 24-30 μM concentrations was demonstrated,^[28] which resembles our results where 30 μM induced the highest caspase-3 cleavage for all leukemia cancer cell lines.

In summary, our data providing evidence on the inhibition of COX-2 and the induction of caspase-3 in Nb4 cells may represent piperlongumine along with the combination of the antioxidant ALA, as a potential therapeutic agent for patients with acute promyelocytic leukemia.

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