Brief communication

Zerumbone induces apoptosis in T-acute lymphoblastic leukemia cells

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ABSTRACT

Zerumbone (ZER) is a potential anticancer natural compound, isolated from Zingiber zerumbet Smith. In this investigation, the anticancer properties of ZER were evaluated on cancer cells of T-acute lymphoblastic leukemia, CEM-ss. The results showed that ZER has cytotoxic effect against CEM-ss cells with an IC50 of 8.4 ± 1.9 μg/ml (coefficient of variation < 30%). Comparatively, 5-fluorouracil (positive control), imposed an inhibitory effect on CEM-ss cells with an IC50 of 1.9 ± 0.06 μg/ml. Scanning electron microscopy (SEM) results revealed abnormalities such as membrane blebbing, holes and cytoplasmic extrusions, all of which are characteristics of apoptosis. In addition, ZER has increased the number of TUNEL-positive stain and the cellular level of caspase-3, the hallmarks of apoptosis, on treated CEM-ss cells. It could be concluded that, ZER was able to produce apoptosis on T-acute lymphoblastic leukemia, CEM-ss. The current findings suggest that ZER might be helpful for improving the usefulness of anticancer agents in the therapy of leukemia.

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1. Introduction

Leukemia is the most common cancer occurring among Malaysian children. Treatment of leukemia includes radiotherapy, chemotherapy, hormonal therapy, immune-intervention and symptomatic and supportive therapy [1]. A dedicated attention in anticancer drugs of herbal origin has developed recently. In Southeast Asia, the rhizomes of Zingiber zerumbet Smith (wild ginger) are employed in traditional medicine. ZER, the major compound in wild ginger, inhibits the proliferation of neoplastic colon, breast, cervix, ovary and liver cells [2,3]. ZER is selective towards cancer cells compared to normal cells [3]. Furthermore, the compound has shown potential in vivo chemo-preventive properties on induced skin, cervix and colon cancers in rodents models. In addition, ZER has shown other biological activities that included anti-cyclooxygenase-2, suppression of free radical generation, modulation of iNOS expression, TNF-a release, down-regulation of IL-1β, and NF-κB and anti-HIV. The biological activities of ZER are distinctively different from those of α-humulene, in which, the later lacks the carbonyl group at the 8-position, as found in ZER [2–4]. On the hand, ZER was found to inhibit the proliferation of acute promyelocytic leukemia cell line, NB4 [5]. ZER was also found not to be clastogenic to cultured human peripheral lymphocytes [6]. There is no literature available on the mechanism of ZER and its effects on T-acute lymphoblastic leukemia cells (CEM-ss), CEM cells are a line of lymphoblastic cells originally derived from a child with acute lymphoblastic leukemia [7]. Therefore, the current study was designed to investigate the anticancer properties of ZER on CEM-ss.

2. Materials and methods

2.1. Anti-proliferative activities of ZER and 5-fluorouracil

Pure ZER crystals were prepared from fresh Z. zerumbet rhizome extract according to the method describe earlier [2]. Purity and molecular weight of ZER were determined using HPLC and LC–MS, respectively. Cytotoxicity of ZER (1–100 μg/ml) on CEM-ss (NSH, USA) was assessed using MTT assay [8]. To validate the experimental settings of this assay, CEM-ss cells were also treated with 5-fluorouracil, as a positive anti-neoplastic agent. Cytotoxicity of 5-fluorouracil on human T-lymphoblast leukemia cells was reported previously [9].

2.2. Exterior ultrastructural effects of ZER on CEM-ss cells

CEM-ss cells were treated with the IC50 of ZER for 0, 24, and 48 h. Harvested cells were fixed, washed in sodium cacodylate buffer and post-fixed in 1% Osmium tetroxide at 4 °C. The specimens were then washed, dehydrated in ascending grades of acetone and brought to critical point of drying by the critical point drier (CPD 030, Bal-TEC, Switzerland). The cells were affixed to a metal SEM stub and sputter coated in gold by using SEM coating unit (E100 Polaror, England). The coated specimens were viewed using scanning electron microscopy (JOEL 64000 Japan) at accelerating voltage of 15–25 kV.

2.3. TUNEL assay

The mode of cell death induced by ZER was determined by morphological observations done with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay using the Apoptotic Detection Kit (Promega Inc., USA) and Zeiss confocal fluorescent microscope (LSM 70). DNAase was used as positive

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Cytotoxicity of zerumbone treated CEM-ss after 72 h treatment as assessed by MTT assay. The IC50 for zerumbone is 8.4 ± 1.9 μg/ml. The results are the mean ± SEM of three separate experiments; *P* < 0.05 vs. control.

**3. Results and discussion**

**3.1. ZER inhibits the proliferation of CEM-ss leukemia cells**

The MTT is a regular and standard colorimetric assay for measuring cell viability. As shown in Fig. 1, ZER exhibited cytotoxic effects against CEM-ss cells with an IC50 of 8.4 ± 1.9 μg/ml (Fig. 1A). The American National Cancer Institute guidelines set the limit of activity for new drugs at a 50% inhibition (IC50) of proliferation of less than 30 μg/ml. 5-Fluorouracil imposed an inhibitory effect on CEM-ss cells with an IC50 value of 1.94 ± 0.06 μg/ml (Fig. 1B). Sensitivity of CEM-ss to 5-fluorouracil indicates that the data obtained for ZER can be accepted. DMSO as negative control did not show any inhibition towards CEM-ss.

![Fig. 1](image1.png)

**Fig. 1.** Cytotoxicity of zerumbone treated CEM-ss after 72 h treatment as assessed by MTT assay. The IC50 for zerumbone is 8.4 ± 1.9 μg/ml. The results are the mean ± SEM of three separate experiments; *P* < 0.05 vs. control.

**2.4. Colorimetric assays of caspase-3**

This assay was performed using commercial kit of ApoTarget™ (BioSource International, Inc. USA). 2 × 10⁶ cells were treated with IC50 of ZER and incubated for 0, 24 and 48 h. The samples were read at 405 nm in a microplate reader (TECAN, Sunrise™, Männedorf, Switzerland). Data was presented as optical density (405 nm; mean ± SD).

**2.5. Statistical analysis**

Statistical analyses were done using SPSS version 16.0 (SPSS Inc., Chicago, USA). Data have been initially evaluated for homogeneity of variance and normality. Probability values of less than alpha (0.05) were considered statistically significant.

![Fig. 2](image2.png)

**Fig. 2.** SEM electronmicrographs of surface ultrastructural characteristics of CEM-ss cells treated with ZER in time dependent manner. (A) The characteristic of untreated CEM-ss cells’ surface showing the restoration of a typical morphological feature of a cancer cell. (B and C) ZER-treated CEM-ss cells showed distinct morphological changes corresponding to typical apoptosis in a time dependent manner.
3.2. ZER produces ultrastructural features of apoptosis in CEM-ss

Till today, the microscopic examination has been the gold standard for the most precise detection of apoptosis [8]. Interpretation of SEM electromicrographs showed idiosyncratic cytomorphological alterations corresponding to a typical morphology of apoptosis, including cell shrinkage, membrane blebbing and formation of apoptotic bodies (Fig. 2). The presence of partly degraded apoptotic bodies around the cell cytoplasm was also evident. These apoptotic effects were found to be time correlated phenomena and this was noticed when considering the number of blebs formation (cytoplasmic extension) as an indicator of cell death via apoptosis. On the contrary, untreated CEM-ss cells showed well preserved morphology (Fig. 2A).

3.3. DNA internucleosomal degradation in CEM-ss treated with zerumbone

DNA fragmentation in ZER-treated CEM-ss cells was detected using TUNEL assay. Apoptotic endonucleases degrade chromosomal DNA during programmed cell death [5]. TUNEL-positive cells were observed after ZER treatments are shown in Fig. 3. The significant increase in the number of apoptotic cells with fragmented DNA was observed after treatment. In addition, significant ($P < 0.05$) time depended increase of fragmented DNA was observed at each time point (Fig. 4). Similar kinds of observations were noted previously using TUNEL assay when ZER induces apoptosis in cervical cancer cells [2].

Fig. 3. TUNEL assay on CEM-ss cells after treatment with ZER. Cells were viewed under Zeiss confocal fluorescent microscope (LSM 70). (A) Untreated CEM-ss cells showing nearly absence of green fluorescent color; (B) ZER-treated CEM-ss cells (24 h) depicting TUNEL-positive stain; (C) Higher number of TUNEL-positive cells was observed after treatment of zerumbone for 48 h.

Fig. 4. Quantification of TUNEL results. ANOVA showed a significant difference ($P < 0.05$) between treated and untreated cells in TUNEL-positive cells.

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Fig. 5. The colorimetric assay of caspase-3 in T-acute lymphoblastic leukemia cells (CEM-ss) treated and untreated with ZER (IC$_{50}$) for 24 and 48 h. ANOVA showed a significant difference ($P < 0.05$) between treated and untreated cells in the activity of caspase-3.
3.4. ZER elevated the cellular level caspase-3 in CEM-ss

As shown in Fig. 5, ZER significantly (Independent t-test, \(P < 0.05\)) stimulated caspase-3, the hallmark enzyme of apoptosis. Caspase-3 enzyme is higher in treated CEM-ss cells as compared to non-treated cells. Caspase-3 level in cancer cells could be measured \textit{in vitro} using colorimetric assay which can be detected at 405 nm [8]. At this moment of writing, this study is the first to demonstrate that caspase-3 activation is required for ZER-induced apoptosis in T-acute lymphoblastic leukemia. It has been reported that ZER inhibits the proliferation of cancer cells, while having less effect on normal-cell proliferation. The \(\alpha,\beta\)-unsaturated carbonyl group in ZER effectively removes the intracellular GSH (glutathione) by forming a Michael adduct with it, thereby raising intracellular redox potential \(E\), which in turn stops the proliferation of the cancer cells. However, average intracellular redox potential of normal cells is different from cancer cells; this difference could explain the weaker effect of ZER on the proliferation of normal cells [3,10].

4. Conclusion

In conclusion, we demonstrated here that ZER inhibits the proliferation of T-acute lymphoblastic leukemia cells (CEM-ss) via the induction by apoptosis. Taken together, the present results suggest that ZER may possess potential as a novel therapeutic agent in the treatment of leukemia.

Conflict of interest statement

All authors declare no conflict of interest.

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