

Zerumbone Exerts Antiproliferative Activity via Apoptosis on HepG2 Cells

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Abstract

Zerumbone, a cytotoxic component isolated from *Zingiber zerumbet* Smith, significantly displayed antiproliferative effect towards human cancer cell lines including the human liver cancer HepG2 cell line (IC_{50} of $3.45 \pm 0.026 \mu\text{g/ml}$), human breast cancer MCF-7 cell line (IC_{50} of $3.73 \pm 0.085 \mu\text{g/ml}$), human ovarian cancer Caov-3 cell line (IC_{50} of $4.73 \pm 0.052 \mu\text{g/ml}$) and human cervix cancer HeLa cell line (IC_{50} of $5.43 \pm 0.033 \mu\text{g/ml}$). The action of zerumbone appeared to be cytospecific as its effect on the proliferation of non-malignant Chang liver cells generated IC_{50} value that was much higher than that obtained for all zerumbone-treated cancer cell lines ($10.96 \pm 0.059 \mu\text{g/ml}$). The antiproliferative effect of zerumbone was also shown to occur via apoptosis. The extent of DNA fragmentation, evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay, showed that zerumbone significantly increased apoptosis of the HepG2 cells in a time-course manner and that its effect was generally more potent than cisplatin.

Keywords: zerumbone, antiproliferative effect, apoptosis, *Zingiber zerumbet* Smith, HepG2

Introduction

In the normal state of cell growth, cell division must be counterbalanced by cell death [1]. The death of cells is physiologically important. It is an actively programmed death of cells known as apoptosis, a term introduced by Kerr to describe a form of hepatocellular carcinoma cell death [2]. Sensitization of cancer cells to drug-induced apoptosis has become an important strategy in overcoming carcinogenesis. Thus, a great deal of research has been turned towards novel chemotherapeutic drugs from the plant kingdom in search of cancer inhibitors and cures. Pezzuto reported that the bioactive components obtained from herbal plants have high potential in preventing and controlling carcinogenesis [3].

In Asia, medicinal herbs are used as treatment for various ailments including malignancies [4]. The Zingiberaceae family is most frequently used as raw material for making various traditional medicine formulations that are commonly sold in the market [5, 6]. Scientific research towards *Zingiber zerumbet* proved that it is mainly modulated by its component, zerumbone, which is the main cytotoxic compound that constitutes about 37% of the whole *Z. zerumbet* content [7].

Zerumbone has been found to exert anti-tumour and anti-inflammatory effects [8, 9]. It was also found to inhibit the proliferation of human colonic adenocarcinoma cell lines in a dose dependent manner but its effect was less effective towards the growth of normal human dermal and colon fibroblasts [10]. It was reported that inhibition of the Epstein-Barr virus early antigen activation which

was induced by tumour-promoters *in vitro* correlated well with the zerumbone anti-tumour promoting effect *in vivo* [9, 11].

In the present study, the effect of zerumbone on the proliferation of several cancer cell lines was studied. The mode of cell death induced by the cytotoxic compound was also determined by morphological observation performed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), penicillin, propidium iodide, streptomycin, fungizon, miramycin and trypsin-EDTA were bought from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO BRL (Gaithersburg, MD). TUNEL Kit was purchased from Promega (Madison, WI). All other chemicals used were of the highest pure grade available. Cell culture plasticware were from Nunc Co. (Denmark). Zerumbone was provided by Prof. Dr. Hasnah from Universiti Teknologi Malaysia.

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Cell Culture

Human liver cancer cell line (HepG2), human carcinoma mammary cell line (MCF-7), human ovarian cancer cell line (Caov-3), human cervix cancer cell line (HeLa), normal cells of Chang liver and Vero were obtained from American Type Cell Culture Collection (ATCC), Maryland, USA. All cultured cells were maintained in the logarithmic phase of growth in DMEM supplemented with 10% foetal bovine serum (GIBCO BRL), penicillin-streptomycin, fungizon and miramycin at 37°C in a humidified incubator with 5% CO₂ and 95% air. Cultures were regularly examined using an inverted microscope.

Antiproliferative assay

Trypsinised cells were counted using a haemocytometer and 1×10^5 cells were plated in a 96-well microtiter plate. After an overnight incubation to allow attachment, medium was changed and 0.2 ml of new supplemented medium was added to each well. Cells were then treated with 2 μ l zerumbone in a dose dependent-manner and incubated at 37°C, 5% CO₂ for 72 hours. DMSO (0.1%) and cisplatin were respectively used as negative and positive treatment controls. Each concentration of the compounds was assayed in triplicates. Antiproliferative effect of zerumbone was monitored employing the methylene blue assay [13]. Glutaraldehyde was added to each well to fix the cells. Wells containing only fixed cells were washed twice with 0.15 M NaCl, and were stained with 0.1 ml 0.05% methylene blue for 15 minutes. To aid spectrophotometric analysis for quantitative determination of viable cells, 0.2 ml of 0.03 M HCl was added to each well for viability measurements. The absorbance was measured at wavelength of 660 nm and the proportion of surviving cells was calculated by dividing the average of non-treated wells (control of a control). Statistical analysis was done using the t-test (Microsoft Excel).

TUNEL assay

The mode of cell death induced by zerumbone was determined by morphological observations done with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay. Cells were grown on microscope slides and were treated with zerumbone based on the IC₅₀ value obtained from the antiproliferative assay. The fragmented DNA of apoptotic cells was quantified by TUNEL using the Apoptotic Detection Kit (Promega Inc., USA). Briefly, the cells were fixed with 4% methanol-free paraformaldehyde at 4°C and washed with phosphate-buffered saline (PBS) for 30 minutes. Equilibrium buffer (0.1 ml) was added to each slides and covered with parafilm for 10 minutes at 37°C. A mixture of 1 μ l TdT enzyme, 5 μ l nucleotide mix and 45 μ l equilibrium buffer was prepared in the dark and 50 μ l of the mixture was added onto each slide. Slides were incubated in the dark for 1 or 2 hours at 37°C. SSC (2X) was added for 15 minutes at room

temperature to stop the TdT enzyme's reaction. The unbound fluorescent-12-dUTP was removed by washing with PBS. The slides were then immersed in propidium iodide for 15 minutes in the dark to stain the cells. Slides were dried after rinsing with deionized water and cover slips were later overlaid on the cell area of the slides. This assay detects only apoptotic cells when examined through the Zeiss fluorescent microscope.

Results

Antiproliferative Assay

Figure 1 shows that zerumbone was able to exert anti-proliferative effect towards most of the human cancer cell lines tested. The IC₅₀ values, which are the concentrations of zerumbone required for 50% inhibition towards HepG2, Caov-3 and MCF-7 cell viability were $3.45 \pm 0.026 \mu\text{g/ml}$, $4.73 \pm 0.052 \mu\text{g/ml}$ and $3.73 \pm 0.085 \mu\text{g/ml}$, respectively. The highest IC₅₀ value obtained from the effect of zerumbone was on HeLa cells, i.e., $5.43 \pm 0.033 \mu\text{g/ml}$. This bioactive compound also inhibited the proliferation of the non-malignant Chang liver cells with an IC₅₀ value of $10.96 \pm 0.059 \mu\text{g/ml}$.

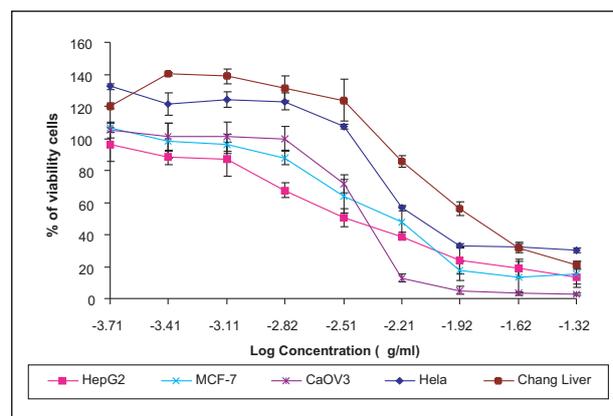


Figure 1: Effects of zerumbone on cell viability of various cancer cell lines and non-cancer cells. Treatment of HepG2, MCF-7, Caov-3 and HeLa cell lines with zerumbone significantly reduced the number of viable cells with IC₅₀ values less than 5 $\mu\text{g/ml}$. Non-malignant Chang liver cells were also affected but the IC₅₀ was generally higher than that obtained for the malignant cell lines (see Table 1 for summary of IC₅₀ values).

For comparison and positive control, cisplatin, a drug with antineoplastic activity was used in this study. Cisplatin is used widely in the treatment of ovarian, bladder and testicular cancers. Our studies demonstrated that cisplatin imposed inhibitory effects on HepG2 and Caov-3 cells with IC₅₀ values of $7.23 \pm 0.036 \mu\text{g/ml}$ and $6.92 \pm 0.06 \mu\text{g/ml}$, respectively. However, the IC₅₀ value towards MCF-7 cell viability exceeded 20 $\mu\text{g/ml}$ ($23.99 \pm 0.059 \mu\text{g/ml}$). Cisplatin was also found to be effective towards the non malignant Vero and Chang liver cells, with IC₅₀ values of $9.06 \pm 0.044 \mu\text{g/ml}$ and 7.08 ± 0.073

$\mu\text{g/ml}$, respectively (Figure 2). Table 1 demonstrates the summary of IC_{50} values obtained from cisplatin and zerumbone treated malignant and non-malignant cells.

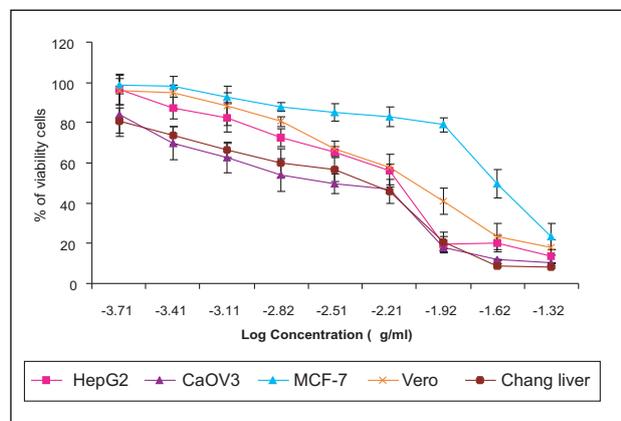


Figure 2: Effects of cisplatin on cell viability of cancer and non-cancer cell lines. The effectiveness of cisplatin on HepG2 cells and non-malignant Chang liver cells did not significantly differ since the IC_{50} values obtained for the malignant and non-malignant cells were both $7 \mu\text{g/ml}$ (see Table 1 for summary of IC_{50} values).

TUNEL Assay

To determine the mode of cell killing by zerumbone, the TUNEL assay was performed to detect only apoptotic cells. In apoptosis, the 3'OH ends of fragmented DNA generated may be labeled with fluorescence-12-dUTP using the enzyme terminal deoxynucleotidyl transferase (TdT). Cells that die of necrosis are not labeled by TdT as there

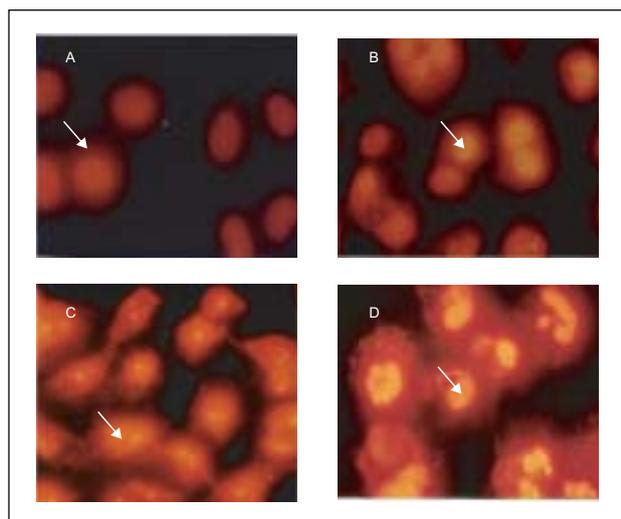


Figure 3: Treatment of HepG2 cells with zerumbone. HepG2 cells were treated with $3.45 \mu\text{g/ml}$ zerumbone for 24 (B), 48 (C) and 72 (D) hours. DMSO treated HepG2 cells served as negative control (A) and thus gave TUNEL-negative results indicating less apoptotic signal. Arrows indicate cells with fragmented DNA due to apoptosis which occurred actively at beginning of the treatment. The presence of apoptotic bodies was observed after 72 hours of treatment. Magnification: 1000X.

are no free 3'OH DNA ends generated. Zerumbone treated-HepG2 cells at 24 hours exhibited chromatin condensation and DNA fragmentation, which are characteristics of early apoptotic cells (Figure 3). More fluorescence TdT-binding occurred at 48 hours of treatment as shown by increase in the intensity of yellow fluorescence. This indicates that more DNA was fragmented and the cells were actively undergoing apoptosis (almost 80%). At 72 hours of treatment, most of the HepG2 cells showed membrane blebbing with condensation of chromatin and presence of apoptotic bodies.

When HepG2 cells were treated with cisplatin, similar results were observed (Figure 4). However, the yellow fluorescence intensity was not as bright as that detected in the zerumbone-treated HepG2 cells. In case of the negative control, HepG2 cells treated with DMSO appeared reddish and no fluorescence was detected in the nuclei, due to the absence of fragmented DNA.

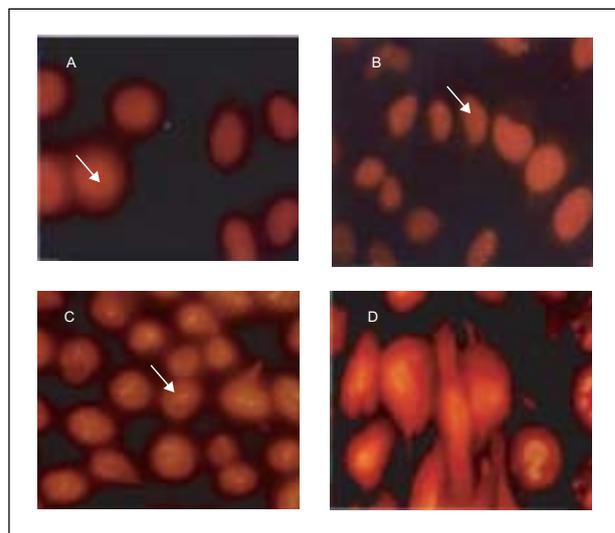


Figure 4: Treatment of HepG2 cells with cisplatin. HepG2 cells were treated with $3.45 \mu\text{g/ml}$ cisplatin for 24 (B), 48 (C) and 72 (D) hours. DMSO treated HepG2 cells served as negative control (A) and thus gave TUNEL-negative results. Arrows indicated cell death via apoptosis. The intensity of yellow fluorescence was lower than the fluorescence observed in zerumbone-treated cells. Magnification: 1000X.

Figure 5 shows the apoptotic index of cultured HepG2 cells treated with zerumbone and cisplatin. In the presence of $3.45 \mu\text{g/ml}$ of zerumbone, the percentages of apoptotic cells increased in a time-course manner with the apoptotic cells representing more than 50% of the total cultured cells at 24 hours and approximately 80% of the cultured cells died of apoptosis by 48 hours. Meanwhile, cells that were treated with $3.45 \mu\text{g/ml}$ cisplatin demonstrated a score of more than 40% of apoptotic cells at 24 hours, which increased gradually with prolonged duration of treatment. More than 70% of the cells had died via apoptosis at 48 hours, and by 72 hours, 80% of the cells had died of apoptosis. Untreated control cells showed only 6% of cell death via apoptosis.

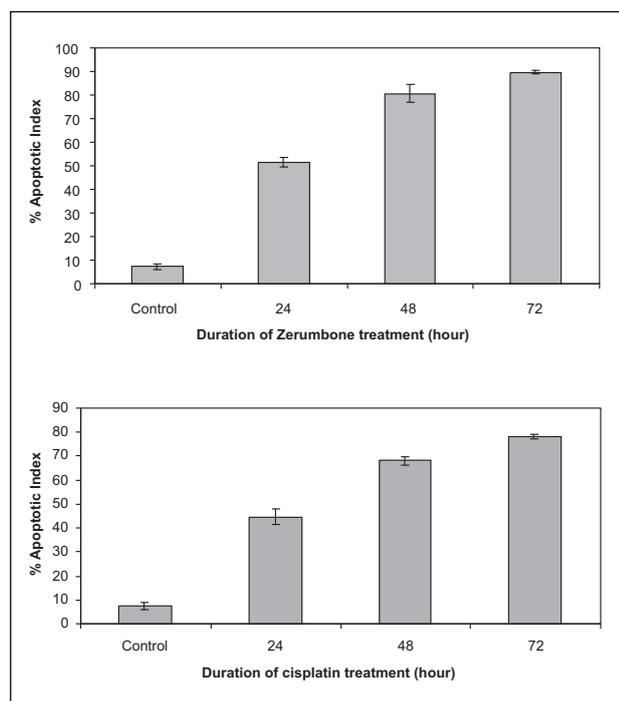


Figure 5: Apoptotic index of cultured HepG2 cells treated with zerumbone and cisplatin. Percentages of HepG2 cell death via apoptosis increased significantly in a time-dependent manner after treatment with (A) zerumbone and (B) cisplatin.

Discussion

In patients with malignant tumours, therapeutic strategies are aimed at equilibrating the imbalance between proliferation and degeneration [13]. The data obtained in this study indicated that zerumbone from the family of Zingiberaceae strongly exerts antiproliferative effect and significantly reduced viability of HepG2, Caov-3, MCF-7 and HeLa cell lines in a dose dependent manner. Although zerumbone was found to also inhibit the proliferation of non-malignant Chang liver cells, the IC_{50} value generated was much higher than that obtained for all the zerumbone-treated cancer cell lines (Table 1).

Table 1: IC_{50} values of cell lines treated with zerumbone and cisplatin.

Cell lines	IC_{50} values ($\mu\text{g/ml}$)	
	zerumbone	cisplatin
HepG2	$3.45 \pm 0.026^{**}$	7.23 ± 0.036
MCF-7	$3.73 \pm 0.085^{**}$	$23.99 \pm 0.059^{**}$
Caov-3	$4.73 \pm 0.052^{**}$	6.92 ± 0.06
HeLa	$5.43 \pm 0.033^{**}$	-
Chang	10.96 ± 0.059	7.08 ± 0.073
Vero	-	9.06 ± 0.044

**denotes value of significance difference ($p < 0.0005$) compared to the negative control DMSO-treated cells.

Similarly, Hoffmann *et al.* have earlier shown that an appropriate dose of zerumbone induced a high intracellular redox potential which stopped proliferation of cancer cells but not the normal cells [14]. This was also observed by Murakami *et al.* who reported that zerumbone inhibited the proliferation of human colonic adenocarcinoma cell line in a dose dependent manner while the growth of normal human dermal (2F0-C25) cells was less affected [10]. Thus, the effects of zerumbone appeared specific towards tumour cells.

Unlike zerumbone, the effect of cisplatin, a cytotoxic compound currently used for treatments of ovarian, bladder and testicular cancers, was not cytospecific since its antiproliferative effect was towards both cancer and normal cells. Several reports have indicated that the nephrotoxic effect of cisplatin was still a common adverse effect in both adults and children even with the use of hyperhydration and other protective measures [15-17]. Kim *et al.* have also reported that cisplatin can cause damage towards liver and kidney [18, 19]. Patients undergoing cisplatin treatment experienced side effects such as emesis, loss of hearing, pressure towards bones, and neurotoxicity [20]. Binding of cisplatin towards DNA formed cisplatin-DNA complex, which caused unnatural changes to its conformation.

Our data indicated that cisplatin treatment generated low IC_{50} values towards normal Chang liver and Vero cells (Table 1). However, its effect towards the cancer cell lines, especially on the HepG2 cells, showed that it was not as effective as zerumbone. In comparison to the IC_{50} of cisplatin-treated Chang liver cells (IC_{50} of $7.08 \pm 0.073 \mu\text{g/ml}$), the IC_{50} value of zerumbone towards the HepG2 cells was twice lower ($3.45 \pm 0.026 \mu\text{g/ml}$). Besides, zerumbone was also less cytotoxic towards normal Chang liver cells (IC_{50} of $10.96 \pm 0.059 \mu\text{g/ml}$) compared to cisplatin-treated normal Chang liver cells (IC_{50} $7.08 \pm 0.073 \mu\text{g/ml}$). In case of the HepG2 cells, cisplatin was found to inhibit their proliferation of at almost the same concentration ($7.23 \mu\text{g/ml}$) as that of zerumbone. Therefore, at the concentration of $7.08 \mu\text{g/ml}$, cisplatin not only killed the HepG2 cells but also normal Chang liver cells. When taken together, these findings together with those that were previously reported suggest that zerumbone is generally a better reagent to use for inhibition of cancer cells compared to cisplatin.

To confirm that zerumbone-treated cell death occurred via apoptosis, the extent of DNA fragmentation was analyzed using calculated Apoptotic Index [AI]. AI is described as the percentage of apoptotic cells and apoptotic bodies within the overall population of total cells [21]. When HepG2 cells were treated with zerumbone ($3.45 \mu\text{g/ml}$), more than 50% of the TUNEL-positive cells were detected at 24 hours of treatment. Gavrielli *et al.* reported that in the early process of apoptosis, DNA fragmentation occurs at the periphery of the nucleus within minutes [22] while lysosomal

degradation ended within hours depending on the types of cell and tissue [1, 23]. In the present study, this can be seen in the large increase of apoptotic scores of around 80% by 48 hours and 90% after 72 hours of cultured HepG2 cells subjected to zerumbone treatment. Untreated control cells only recorded approximately 6% of apoptotic cells. When HepG2 cells were treated with cisplatin at 3.45 $\mu\text{g}/\text{ml}$, measurable increased in apoptosis occurred within 24 hours of treatment with scores of more than 40%, which increased up to above 60 and 70% at 48 and

72 hours, respectively. The data obtained from the scores also suggested that zerumbone was more effective in inducing apoptosis compared to cisplatin.

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