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 ± 0.026 µg/ml), human breast cancer MCF-7 cell line (IC$_{50}$ of 3.73 ± 0.085 µg/ml), human ovarian cancer Caov-3 cell line (IC$_{50}$ of 4.73 ± 0.052 µg/ml) and human cervix cancer HeLa cell line (IC$_{50}$ of 5.43 ± 0.033 µg/ml). The action of zerumbone appeared to be cytoselective 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 ± 0.059 µg/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 tryspin-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.

Antiproliferative assay

Trypsinised cells were counted using a haemocytometer and 1 x 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 µl 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 treatment) 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 IC_{50} values, which are the concentrations of zerumbone required for 50% inhibition towards HepG2, Caov-3 and MCF-7 cell viability were 3.45 ± 0.026 µg/ml, 4.73 ± 0.052 µg/ml and 3.73 ± 0.085 µg/ml, respectively. The highest IC_{50} value obtained from the effect of zerumbone was on HeLa cells, i.e., 5.43 ± 0.033 µg/ml. This bioactive compound also inhibited the proliferation of the non-malignant Chang liver cells with an IC_{50} value of 10.96 ± 0.059 µg/ml.

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 IC_{50} values of 7.23 ± 0.036 µg/ml and 6.92 ± 0.06 µg/ml, respectively. However, the IC_{50} value towards MCF-7 cell viability exceeded 20 µg/ml (23.99 ± 0.059 µg/ml). Cisplatin was also found to be effective towards the non-malignant Vero and Chang liver cells, with IC_{50} values of 9.06 ± 0.044 µg/ml and 7.08 ± 0.073 µg/ml.
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µg/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 µg/ml (see Table 1 for summary of IC$_{50}$ values).](image)

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 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 3: Treatment of HepG2 cells with zerumbone. HepG2 cells were treated with 3.45 µg/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. 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.](image)

![Figure 4: Treatment of HepG2 cells with cisplatin. HepG2 cells were treated with 3.45 µg/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.](image)

Figure 5 shows the apoptotic index of cultured HepG2 cells treated with zerumbone and cisplatin. In the presence of 3.45 µg/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 µg/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.
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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 IC50 value generated was much higher than that obtained for all the zerumbone-treated cancer cell lines (Table 1).

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 cytoselective 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 damaged towards liver and kidney [18, 19]. Patients undergoing cisplatin treatment experienced side effects such as emesis, lost 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 IC50 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 IC50 of cisplatin-treated Chang liver cells (IC50 of 7.08 ± 0.073 µg/ml), the IC50 value of zerumbone towards the HepG2 cells was twice lower (3.45 ± 0.026 µg/ml). Besides, zerumbone was also less cytotoxic towards normal Chang liver cells (IC50 of 10.96 ± 0.059 µg/ml) compared to cisplatin-treated normal Chang liver cells (IC50 7.08 ± 0.073 µg/ml). In case of the HepG2 cells, cisplatin was found to inhibit their proliferation of at almost the same concentration (7.23 µg/ml) as that of zerumbone. Therefore, at the concentration of 7.08 µg/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 µg/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

![Graph](image1.png)

**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.**

![Graph](image2.png)

**Table 1: IC50 values of cell lines treated with zerumbone and cisplatin.**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>zerumbone</th>
<th>cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>3.45 ± 0.026**</td>
<td>7.23 ± 0.036</td>
</tr>
<tr>
<td>MCF-7</td>
<td>3.73 ± 0.085**</td>
<td>23.99 ± 0.059**</td>
</tr>
<tr>
<td>Caov-3</td>
<td>4.73 ± 0.052**</td>
<td>6.92 ± 0.06</td>
</tr>
<tr>
<td>HeLa</td>
<td>5.43 ± 0.033**</td>
<td>-</td>
</tr>
<tr>
<td>Chang</td>
<td>10.96 ± 0.059</td>
<td>7.08 ± 0.073</td>
</tr>
<tr>
<td>Vero</td>
<td>-</td>
<td>9.06 ± 0.044</td>
</tr>
</tbody>
</table>

**denotes value of significance difference (p<0.0005) compared to the negative control DMSO-treated cells.
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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 $3.45 \mu g/ml$, 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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References