UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECTS OF ZERUMBONE ON OVARIAN AND CERVICAL CANCER CELL LINES

ZETTY NADIA BINTI MOHD ZAIN

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CYTOTOXIC EFFECTS OF ZERUMBONE ON OVARIAN AND CERVICAL CANCER CELL LINES

By

ZETTY NADIA BINTI MOHD ZAIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

April 2005
This thesis is dedicated to my loving family who has been supporting me.

Through the good and the bad times they have been whatever I needed.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

CYTOTOXICITY EFFECT OF ZERUMBONE ON OVARIAN AND CERVICAL CANCER CELL LINES

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April 2005

Chairman: Dr Ahmad Bustamam Abdul, PhD.
Faculty: Medical and Health Sciences

Globally, ovarian cancer is the fifth most common cancer among women that affects approximately 1 in 75 women in the developed countries. Over 75% of cases were presented at an advanced stage, with disease spread beyond the ovaries. To date, cervical cancer in women remains a major problem with about 400,000 new cases per year and almost 250,000 reported deaths. However, this disease affects predominantly poor women in underdeveloped countries. It is estimated that 60% of the global market for anticancer and anti-infectious drugs or those under clinical trial are of natural origin. Zerumbone, a sesquiterpene compound isolated from the rhizomes Zingiber zerumbet was shown to suppress TNF-α release and also induces apoptosis in a variety of human colonic adenocarcinoma cell lines. In this current study, the chemotherapeutic potential of zerumbone in cervical cancer (HeLa) and ovarian cancer (Caov-3) cell lines of human origin was evaluated together with cisplatin, a commercially used drug currently used for treating ovarian and cervical...
cancers. Exposure of both cancer cells to a range of zermulone concentrations demonstrated growth inhibition in both cancer cells at a dose-dependent manner. The IC$_{50}$ values, determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reduction assay were as follows: zermulone; Caov-3, 24.0 µM (5.2 µg/ml), HeLa, 20.7 µM (4.5 µg/ml) and cisplatin; Caov-3, 3.7 µM (1.1 µg/ml), HeLa, 5.3 µM (1.6 µg/ml). Laser scanning confocal microscopy following AO/PI staining were used to examine morphological changes of both cancer cells after zermulone and cisplatin treatment. Apoptotic features that included membrane blebbing and nucleus condensation were evident in both treated cancer cells. Following this, TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was conducted to confirm apoptosis. The studies conducted seems to suggest that zermulone induce cell death by stimulating apoptosis better than cisplatin, based on significantly higher percentage of apoptotic cells in zermulone treated cancer cells as compared to cisplatin. In addition, zermulone and cisplatin arrests cancer cells at G$_{2}$/M phase as analyzed by flow cytometry. Abnormal synthesis of IL-6 appears to contribute to the pathogenesis of several kinds of diseases and the constitutive production of IL-6 has been implicated in malignant diseases. Increased levels of IL-6 indicated the aggressiveness of a disease. IL-6 is suggested to provide prognostic value based on its role as a cancer cell growth factor. The effects of zermulone on IL-6 levels were studied using a human base ELISA. The results indicated that zermulone significantly decreased the levels of IL-6 secreted by both cancer cells. However, membrane-bound IL-6 receptor is still intact after zermulone treatment as demonstrated using immunofluorescence technique. This study concludes that the
compound, zerumbone inhibits both cancer cells growth through the induction of apoptosis, arrests cell cycle at G2/M phase and inhibits the secretion levels of IL-6 in both cancer cells. Therefore, zerumbone is a potential candidate as a useful chemotherapeutic agent in treating both cervical and ovarian cancers in future.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN SITOTOKSIK ZERUMBONE TERHADAP SEL-SEL SELANJAR KANSER OVARI DAN KANSER SERVIKS

Oleh

ZETTY NADIA BINTI MOHD ZAIN

November 2004

Pengerusi: Dr Ahmad Bustamam Abdul, PhD.
Fakulti: Perubatan dan Sains Kesihatan

Secara global, kanser ovari ialah kanser yang kelima biasa berlaku di kalangan wanita dan dialami oleh 1 daripada 75 wanita di negara maju. Lebih daripada 75% kes adalah di peringkat akhir di mana kanser telah merebak ke semua bahagian ovari. Kanser serviks pada wanita merupakan masalah besar dengan 400,000 kes baru dilaporkan setiap tahun dan menyebabkan 250,000 kematian, tetapi dialami oleh lebih ramai wanita miskin di negara kurang membangun. Dijangkakan lebih kurang 60% drug antikanser dan anti-jangkitan baik yang sudah berada di pasaran mahupun percubaan klinikal berasal dari alam semulajadi. Zerumbone, sebatian seskuiterpen yang diasingkan daripada rizom Zingiber zerumbet telah menunjukkan perencatan pengeluaran TNF-α dan merangsang apoptosis pada sel-sel selanjar adenokarsinoma kolon manusia. Di dalam kajian ini, dengan menggunakan sel selanjar kanser serviks (HeLa) dan kanser ovari (Caov-3) manusia, potensi kemoterapeutik zerumbone untuk kanser ovari dan serviks dikaji bersama cisplatin, drug antikanser komersil yang sekarang digunakan untuk kanser ovari dan serviks. Pendedahan sel-sel kepada jutil...
kepekatan zerumbone yang berbeza menghasilkan perencatan pertumbuhan bagi kedua jenis sel kanser dengan bergantung kepada nilai kepekatan. Nilai IC₅₀ yang didapati daripada asai penurunan MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-
tetrazolium bromide) adalah seperti berikut: zerumbone; Caov-3, 24.0 μM (5.2 μg/ml), HeLa, 20.7 μM (4.5 μg/ml) and cisplatin; Caov-3, 3.7 μM (1.1 μg/ml), HeLa, 5.3 μM (1.6 μg/ml). Kajian morfologi menggunakan mikroskop fasa terbalik dan mikroskop imbasan laser konfokal selepas perwarnaan dengan AO/PI dijalankan untuk melihat perubahan morfologi bagi kedua sel kanser selepas rawatan zerumbone dan cisplatin. Ciri-ciri apoptosis seperti gelembung membran dan kondensasi nukleus telah didapati bagi kedua-dua sel selanjar yang dirawat. Seterusnya, asai TUNEL (TdT-mediated dUTP Nick-End Labeling) dijalankan untuk menentukan apoptosis. Menariknya, zerumbone didapati menyebabkan kematian sel dengan merangsang apoptosis lebih baik daripada cisplatin berdasarkan peratusan sel-sel apoptotik yang menunjukkan perbezaan yang signifikan berbanding sel-sel kanser dengan rawatan zerumbone. tambahan pula, zerumbone dan cisplatin menahan sel-sel kanser di fasa G₂/M seperti yang dianalisis dengan menggunakan ‘flow cytometry’. Sintesis IL-6 yang luar biasa dikatakan menyumbang kepada patogenesis beberapa penyakit dan penghasilan berterusan IL-6 telah diimplikasikan dalam penyakit malignan. Peningkatan tahap IL-6 menunjukkan perebakkan penyakit secara agresif. IL-6 dicadangkan mempunyai nilai prognostik berdasarkan fungsinya sebagai faktor pertumbuhan sel kanser. Kesana zerumbone terhadap tahap IL-6 dikaji menggunakan ELISA. Keputusan yang didapati menunjukkan zerumbone menurunkan tahap IL-6 yang dirembes oleh kedua jenis sel kanser dengan signifikan. Walau bagaimanapun,
reseptor IL-6 yang terdapat di membran masih ada selepas rawatan zerumbone seperti yang telah didemonstrasikan melalui teknik immunofluoresen. Daipada kajian ini, didapati zerumbone merencat pertumbuhan sel-sel kanser dengan merangsang apoptosis, menahan pada fasa G2/M dan merencat tahap IL-6 sel HeLa dan sel Caov-3. Oleh itu, zerumbone dicadangkan mempunyai potensi perubatan untuk terapi kanser serviks dan ovari.
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Last but not least, I would like to thank to people and everyone who has helped me direct or indirectly towards completing this research project.
I certify that an Examination Committee met on 19th April 2005 to conduct the final examination of Zetty Nadia binti Mohd Zain on her Master of Science thesis entitled “Cytotoxic Effects of Zerumbone on HeLa and CaOV3 Cancer Cell Lines” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

**Daud Ahmad Israf Ali, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Fauziah Othman, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Asmah Rahmat, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Onn Hashim, PhD**  
Professor  
Faculty of Medicine  
Universiti Malaya  
(External Examiner)

---

**GULAM RUSUL RAHMAT ALI, PhD**  
Professor/Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 21 JUL 2005
This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Ahmad Bustamam Abdul, Ph.D.
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Muhd. Nazrul Hakim Abdullah, DVPM, Ph.D.
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 AUG 2005
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(ZETTY NADIA BINTI MOHD ZAIN)

Date: 16 SEPTEMBER 2005
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The concentration-response curve of human ovarian cancer cell (Caov-3) derived from MTT cytotoxicity assay performed after 72 hour exposures with zerumbone. Data are presented as means ± the standard deviation of the mean (n = 3). Double asterisks denote a P of <0.01 when compared with control as analyzed by one-way ANOVA.

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Phase contrast microscopy of Caov-3 cells treated with zerumbone and cisplatin at IC50 value after 24 hour (x200 magnification), control (A), zerumbone (B) and cisplatin (C).

Phase contrast microscopy of Caov-3 cells treated with zerumbone and cisplatin at IC50 value after 48 hour (x200 magnification), control (D), zerumbone (E) and cisplatin (F).

Phase contrast microscopy of Caov-3 cells treated with zerumbone and cisplatin at IC50 value after 72 hour (x200 magnification), control (G), zerumbone (H) and cisplatin (I).

Phase contrast microscopy of HeLa cells treated with zerumbone and cisplatin at IC50 value after 24 hour (x200 magnification), control (J), zerumbone (K) and cisplatin (L).

Phase contrast microscopy of HeLa cells treated with zerumbone and cisplatin at IC50 value after 48 hour (x200 magnification), control (M), zerumbone (N) and cisplatin (O).
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Morphological changes of Caov-3 cells following zerumbone and cisplatin IC_{50} value treatment at 24 hour as viewed under laser scanning confocal microscopy after staining with AO/PI. (x1000 magnification), control (A), zerumbone (B), and cisplatin (C). Arrow B: blebbing of the cell membrane, M: cell membrane damage.

Morphological changes of Caov-3 cells following zerumbone and cisplatin treatment at 48 hour as viewed under laser scanning confocal microscopy after staining with AO/PI. (x1000 magnification), control (D), zerumbone (E), and cisplatin (F). Arrow B: blebbing of the cell membrane, N: nuclear condensation with margination and nuclear damage.

Morphological changes of HeLa cells following zerumbone and cisplatin treatment at 24 hour as viewed under laser scanning confocal microscopy after staining with AO/PI. (x1000 magnification), control (G), zerumbone (H), and cisplatin (I). Arrow B: blebbing of the cell membrane.

Morphological changes of HeLa cells following zerumbone and cisplatin treatment at 48 hour as viewed under laser scanning confocal microscopy after staining with AO/PI. (x1000 magnification), control (J), zerumbone (K), and cisplatin (L). Arrow B: blebbing of the cell membrane, N: nuclear condensation with margination and nuclear damage.

Apoptosis detection using TUNEL Assay of Caov-3 cells treated with zerumbone and cisplatin at IC_{50} value after 24 hour as viewed under laser scanning confocal microscopy. (x600 magnification), control (A), zerumbone (B), and cisplatin (C). Turquoise arrow: mitotic cell, White arrow: nucleus fragmentation and chouromatin condensation to the nuclear membrane.

Apoptosis detection using TUNEL Assay of HeLa cells treated with zerumbone and cisplatin at IC_{50} value after 24 hour as viewed under laser scanning confocal microscopy. (x600 magnification), control (D), zerumbone (E), and cisplatin (F). Turquoise arrow: mitotic cell, White arrow: nucleus fragmentation.
Apoptosis detection using TUNEL Assay of Caov-3 cells treated with zerumbone and cisplatin at IC_{50} value after 48 hour as viewed under laser scanning confocal microscopy. (x600 magnification), control (G), zerumbone (H), and cisplatin (I). White arrow: apoptotic bodies.

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Zerumbone and cisplatin induced apoptosis in Caov-3 cells as treated with IC_{50} values for 24 and 48 hour, respectively. Apoptotic cells were quantified manually after TUNEL Assay. Data shown are mean ± SD from duplicate samples. A single asterisk denotes a P of <0.05 when compared with control; Double asterisks denote a P of <0.01 when compared with control as performed by one-way ANOVA.

Zerumbone and cisplatin induced apoptosis in HeLa cells as treated with IC_{50} values for 24 and 48 hour, respectively. Apoptosis was quantified manually after TUNEL Assay. Data shown are mean ± SD from duplicate samples. Double asterisks denote a P of <0.01 when compared with control as performed by one-way ANOVA.

Flow cytometry analysis of Caov-3 cells after 24 hour exposure to 5, 15, 25, 35 and 45 μM of zerumbone. Cell cycle analysis shows an increase in the number of Caov-3 cells in the G2/M phase and a decrease in the G1 phase and S phase. Data shown are mean ± SD from two different experiments. A single asterisk denotes a P of <0.05 when compared with control; Triple asterisks denote a P of <0.001 when compared with control as performed by one-way ANOVA.

Flow cytometry analysis of HeLa cells after 24 hour exposure to 5, 15, 25, 35 and 45 μM of zerumbone. Cell cycle analysis showed an increase in the number of HeLa cells in the G2/M phase and a decrease in the G1 phase and S phase. Data shown are mean ± SD from two different experiments. A single asterisk denotes a P of <0.05 when compared with control; Double asterisks denote a P of <0.01 when compared with control;
Triple asterisks denote a $P$ of $<0.001$ when compared with control as performed by one-way ANOVA.

Flow cytometry analysis of Caov-3 cells after 24 hour exposure to 5 and 15 μM of cisplatin. Cell cycle analysis shows an increase in the number of Caov-3 cells in the G$_2$/M phase. Data shown are mean $\pm$ SD from two different experiments. Double asterisks denote a $P$ of $<0.01$ when compared with control; Triple asterisks denote a $P$ of $<0.001$ when compared with control as performed by one-way ANOVA.

Flow cytometry analysis of HeLa cells after 24 hour exposure to 5 and 15 μM of cisplatin. Cell cycle analysis shows an increase in the number of HeLa cells in the G$_2$/M phase. Data shown are mean $\pm$ SD from two different experiments. A single asterisk denotes a $P$ of $<0.05$ when compared with control; Double asterisks denote a $P$ of $<0.01$ when compared with control; Triple asterisks denote a $P$ of $<0.001$ when compared with control as performed by one-way ANOVA.

Levels of interleukin-6 (IL-6) secreted by human cervical cancer cells (HeLa) after treatment with zerumbone at 24, 48 and 72 hour incubation as assayed by human IL-6 ELISA. Data are presented as means $\pm$ the standard error (SEM) of the mean ($n = 3$). Triple asterisks denote a $P$ of $<0.001$ when compared with control as analyzed by one-way ANOVA.

Levels of interleukin-6 (IL-6) secreted by human ovarian cancer cells (Caov-3) after treatment with zerumbone at 24, 48 and 72 hour incubation as assayed by human IL-6 ELISA. Data are presented as means $\pm$ the standard error (SEM) of the mean ($n = 3$). Double asterisks denote a $P$ of $<0.01$ when compared with control; Triple asterisks denote a $P$ of $<0.001$ when compared with control.

Immunofluorescence detection of IL-6R expression on Caov-3 cells. The IL-6R cytoplasmic localization were observed under fluorescence microscopy (x200 magnification), control (A), zerumbone, 10 μM (B), 20 μM (C) and 30 μM (D).

Immunofluorescence detection of IL-6R expression on HeLa cells. The IL-6R cytoplasmic localization were observed under fluorescence microscopy (x200 magnification), control (E), zerumbone, 10 μM (F), 20 μM (G) and 30 μM (H).
Flow diagram for protocol of DeadEnd™ Fluorometric TUNEL System

Preparation of IL-6 standard dilutions
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<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
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<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
</tr>
<tr>
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<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
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<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilisation</td>
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<tr>
<td>NCI</td>
<td>National Cancer Institute, USA</td>
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<tr>
<td>NNK</td>
<td>4-(methylnitrosamo)-1-(3-pyridyl)-1-butaneone</td>
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<tr>
<td>sIL-6R</td>
<td>soluble interleukin-6 receptor</td>
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<tr>
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</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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